Universidade do Minho
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Biotechnological approaches of crude glycerol use: optimization of citric acid production by *Yarrowia lipolytica*

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Biotechnological approaches of crude glycerol use: optimization of citric acid production by *Yarrowia lipolytica*

Thesis submitted in fulfilment of the requirements for the degree of Ph.D. in Chemical and Biological Engineering

Work developed under supervision of:
Doctor Isabel Maria Pires Belo
Doctor Manuel José Magalhães Gomes Mota

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“All of science is nothing more than the refinement of everyday thinking.”

Albert Einstein

ABSTRACT

The increase of biodiesel production results in an accumulation of crude glycerol, the main byproduct from biodiesel industry. To take advantage of the glycerol surplus, many biotechnological processes are being studied. Crude glycerol can be used as carbon source to produce citric acid by *Yarrowia lipolytica* under nitrogen-limited growth conditions. However, other operational and medium conditions directly affect citric acid production. Yield of citric acid depends upon the concurrent production of other organic acids, for instance isocitric acid, which is strongly dependent of the strain used.

Although there are some works described in the literature, several factors still need to be completely understood and optimized in the production of citric acid using crude glycerol.

To start with, an experimental design, based on Taguchi method was applied to optimize the culture conditions and to evaluate the effect of pH, carbon/nitrogen (C/N) ratio in the medium, oxygen mass transfer rate (OTR) and salts concentration on citric acid production from pure glycerol by two different *Y. lipolytica* strains (W29 (ATCC 20460) and CBS 2073). OTR and pH were the factors, which had more effect on citric acid production. Moreover, a significant interaction between the factors OTR and salts was observed. The optimal conditions were also validated with crude glycerol and the citric acid production was similar for both strains using this low cost substrate. Since, as shown by the Taguchi approach, a high OTR was crucial for citric acid production, it seemed appropriate to further study this matter. Therefore a model describing oxygen volumetric mass transfer coefficient (k_La), in a lab-scale stirred tank bioreactor (STR), as a function of operating conditions (stirring and aeration rates) and cellular density in the citric acid bioprocess, was developed. An empirical correlation was established that fit well in a wide range of operating conditions. As a result, it was found that raising k_La from 7 h^{-1} to 55 h^{-1} the citric acid concentration increased. On the other hand, the increase of dissolved oxygen concentration (DO) up to 60 % using controlled DO, led to an increase of citric acid concentration, reaching identical concentration as obtained at k_La of 55 h^{-1}. This work demonstrated that k_La is an adequate parameter for the optimization and scale-up of citric acid production from crude glycerol by *Y. lipolytica* W29.

Taking into account that oxygen is a crucial parameter in citric acid production by *Y. lipolytica* W29 from crude glycerol, a pressurized and an airlift bioreactor, both reactors associated to high mass transfer efficiency, were used for batch cultures. Increasing air pressure from 1 bar to 2 bar led to an improvement of 40 % in citric acid concentration, whereas in the airlift bioreactor, with an increase from 1 vvm to 1.5 vvm of the aeration rate a 30 % enhancement was attained. Both bioreactor types can be used as alternative ways of improving OTR for citric acid production, leading to important operating costs savings due to less power input.

The simultaneous production of isocitric acid is the major problem of using *Y. lipolytica* strains as citric acid producer. In order to isolate improved strains with reduced isocitric/citric acid ratio and/or enhanced citric acid production, *Y. lipolytica* W29 was treated with ultraviolet (UV)-irradiation and/or ethyl methane sulfonate (EMS). A 76% and 2.2- fold higher concentration yield of citric acid, was obtained with a mutant strain, *Y. lipolytica* UV/EMS-10, isolated after the combined treatment with UV-irradiation and EMS.

RESUMO

O crescimento na produção de biodiesel tem produzido um aumento da quantidade de glicerol bruto disponível, o principal subproduto da indústria do biodiesel. Vários processos biotecnológicos têm sido estudados como alternativas para poder escoar a quantidade de glicerol bruto disponível. O glicerol bruto pode ser utilizado como fonte de carbono na produção de ácido cítrico pela levedura *Yarrowia lipolytica* em condições de crescimento com quantidade limitante de azoto. No entanto, outras condições operacionais, o meio de cultura e a estirpe utilizada podem influenciar o perfil de produção de ácido cítrico. Para maximizar a produção de ácido cítrico é muito importante otimizar todos os factores que possam influenciar a sua produção e também entender como esses factores podem afectar o perfil de produção.

Inicialmente, foi utilizado um desenho experimental, baseado no método de Taguchi, para otimizar as condições de cultura e avaliar o efeito dos fatores pH, razão carbono/azoto (C/N), taxa de transferência de oxigénio (OTR) e a concentração de sais, na produção de ácido cítrico por duas estirpes diferentes de *Y. lipolytica* (W29 (ATCC 20460) e CBS 2073) a partir de glicerol puro. Os fatores OTR e pH foram os que mais influenciaram a produção de ácido cítrico. No entanto observou-se um importante efeito de interação entre fatores, principalmente entre OTR e a concentração de sais. As condições ótimas foram também validadas usando glicerol bruto, sendo a produção de ácido cítrico semelhante para ambas as estirpes estudadas. Uma vez que, através do método Taguchi, ficou comprovada a importância crucial da oxigenação, foi decidido aprofundar esta matéria, tendo-se desenvolvido uma correlação empírica explicativa da relação entre o coeficiente volumétrico de transferência de massa de oxigénio (k_La) num biorreactor de tanque agitado (STR), em função das condições de operação (agitação e taxa de arejamento) e da densidade celular. O aumento do k_La até 55 h^{-1} resultou num aumento na concentração de ácido cítrico. Por outro lado, utilizando oxigénio dissolvido (DO) constante, o aumento de DO até 60 % de saturação levou a um aumento da concentração de ácido cítrico, tendo-se obtido uma concentração semelhante à conseguida para o k_La de 55 h^{-1}. Neste trabalho demonstra-se que o k_La é um fator adequado de otimização e a ter em conta no aumento de escala da produção de ácido cítrico.

Considerando que o oxigénio dissolvido é um parâmetro crucial na produção de ácido cítrico pela *Y. lipolytica* W29 a partir de glicerol bruto foram utilizados dois bioreatores normalmente associados a elevada eficiência de OTR, um bioreator pressurizado e um *airlift*. Um aumento da pressão total de ar até 2 bar melhorou em 40 % a concentração e rendimento em ácido cítrico, enquanto que, no biorreator do tipo *airlift*, um aumento na taxa de arejamento até 1,5 vvm resultou numa melhoria de 30 % para ambos os parâmetros. Ou seja, ambos os biorreatores podem ser usados como alternativa para aumentar OTR na produção de ácido cítrico, levando a importantes poupanças nos custos de operação.

A produção simultânea de ácido isocítrico é o principal problema quando se utiliza estirpes de *Y. lipolytica* na produção de ácido cítrico. Assim, numa tentativa de obter estirpes que apresentem um menor rácio ácido isocítrico/cítrico e/ou uma melhor produção de ácido cítrico, a estirpe *Y. lipolytica* W29 foi sujeita a uma exposição a irradiação ultra violeta (UV) e/ou um mutagénico químico, metano sulfonato de etilo (EMS). Após o tratamento que combinou a irradiação UV com a exposição a EMS, isolou-se a estirpe mutante *Y. lipolytica* UV/EMS-10, que apresentou um aumento de 76 % na concentração de ácido cítrico e um rendimento em ácido cítrico 2,2 vezes maior em comparação com a estirpe parental.

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LIST OF ABBREVIATIONS

a	Interfacial area
ace⁻	Acetate negative
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
c	Constant dependent on the impeller
C	Dissolved oxygen concentration in the liquid ($mg \cdot L^{-1}$)
C_i	Dissolved oxygen concentration in the beginning ($mg \cdot L^{-1}$)
C_0	Dissolved oxygen concentration when aeration is restarted ($mg \cdot L^{-1}$)
*C**	Solubility of oxygen in the liquid ($mg \cdot L^{-1}$)
C/N	Carbon/nitrogen ratio
D_i	Impeller diameter (m)
DAD	Diode array detector
DO	Dissolved oxygen concentration
EMS	Ethyl methanesulfonate
FDA	Food and Drug Administration
F_g	Volumetric gas flow rate ($m^3 \cdot s^{-1}$)
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
ICA/CA	Isocitric/citric acids ratio ($g \cdot g^{-1}$)
k_L	Liquid side mass transfer coefficient ($m \cdot s^{-1}$)
k_La	Oxygen volumetric mass transfer coefficient (h^{-1})
K_T	Constant dependent on the impeller
N	Stirring rate (rps)
N_p	Power number
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NG	N-methyl-N'-nitro-N-nitrosoguanidine
OUR	Oxygen uptake rate ($mg \cdot g^{-1} \cdot h^{-1}$)

OTR	Oxygen mass transfer rate ($mg \cdot L^{-1} \cdot h^{-1}$)
P_g	Power input to the aerated system (W)
P_g'	Power input to the non-aerated system (W)
P	Maximum productivity ($g \cdot L^{-1} \cdot h^{-1}$)
q_{CA}	Maximum specific citric acid productivity ($g \cdot g^{-1} \cdot h^{-1}$)
q_S	Specific substrate consumption rate ($g \cdot g^{-1} \cdot h^{-1}$)
Re	Reynolds number
RI	Refractive Index
ROS	Reactive oxygen species
rpm	Rotation per minute
SCO	Single cell oil
SCP	Single cell protein
SI	Severity index (%)
STR	Stirred tank reactor
t	Time (h)
t_0	Time when aeration is restarted (h)
TCA	Tricarboxylic acid cycle
UV	Ultraviolet
V	Bioreactor working volume (m^3)
v	Liquid viscosity ($kg \cdot m^{-1} \cdot s^{-1}$)
v_s	Superficial gas velocity ($m \cdot s^{-1}$)
vvm	Volume of air per volume of medium per minute
YPD	Yeast extract, peptone, dextrose medium
YPDA	Yeast extract, peptone, dextrose and agar medium
$Y_{CA/S}$	Citric acid yield per substrate mass consumed ($g \cdot g^{-1}$)
$Y_{X/S}$	Cell mass yield per substrate mass consumed ($g \cdot g^{-1}$)

Subscripts

0	Initial condition
CA	Citric acid
i	Condition

S	Substrate
X	Biomass

Greek letters

α	Numerical constant
β	Numerical constant
δ	Numerical constant
ρ	Liquid density ($kg \cdot m^{-3}$)
μ	Maximum specific growth rate (h^{-1})
τ	Probe response time (s)

Remarks:

In general, the International System of Units (SI) was used in this work. Sometimes multiples and sub-multiples of the SI units were also used, as well as other non-SI units but allowed by SI, such as the use of liter to express volume.

Some units not recognized by the SI were also used to express some variables, such as the volume percent (% v/v), mass percent (% w/w) and mass per volume percent (% w/v) to denote the composition of some solutions, the revolutions per minute (rpm) to indicate the agitation rates and the volume of air per volume of reactor per minute (vvm) to designate the aeration rates, due to the usual use in fermentation technology area.

1 MOTIVATION AND OUTLINE

This chapter introduces the background information about the theme of the work, as well as its objectives.

The outline of the thesis and its outputs are also presented.

1.1 CONTEXT AND MOTIVATION

The increasing world population and consequent need of energy made its production a priority. Energy is mainly produced from fossil fuel, a highly polluted material that due to its scarce led to a price demand and consequently to an energetic crisis (Dobson *et al.*, 2012). Also, there is an increasing environmental awareness towards to use of polluting energy sources and a clear shift to the use of friendlier alternatives. These changes pressured scientists and the industry to push forward the development of renewable energy sources (Johnson and Taconi, 2007). Biodiesel, produced from vegetable oils by transesterification with an alcohol, (Meher *et al.*, 2006) has become an alternative source of energy to the fossil fuel, mainly in the transport division, creating this way a new market with exponential growth opportunities (Johnson and Taconi, 2007). In Europe by 2014, biodiesel production capacity was approximately 23 million tons, 2.5 % of which produced by Portugal, according to European Biodiesel Board (see http://www.ebb-eu.org). Biodiesel production increase led to an accumulation of highly glycerol concentrated residual wastes (Chatzifragkou *et al.*, 2011).

Crude glycerol is the main byproduct of biodiesel industry, where 10 kg of biodiesel produced generates 1 kg of glycerol. However, crude glycerol is not a pure and due to presence of impurities, the use of crude glycerol in traditional applications become limited (Johnson and Taconi, 2007; Papanikolaou *et al.*, 2008a). The purification of crude glycerol is not a cost effective process to be used in the chemical, textile, pharmaceutical, cosmetic and food industries (Wang *et al.*, 2001; Amaral *et al.*, 2009). Thus, it becomes necessary to select strategies of recovery and valorization of this byproduct in its unpurified form (Johnson and Taconi, 2007; Çelik *et al.*, 2008; Amaral *et al.*, 2009). Biotechnological conversion of crude glycerol by microbial fermentation into value-added products has been proposed by many authors (Koutinas *et al.*, 2007; André *et al.*, 2010). Indeed, there is a wide diversity of microorganisms able to use glycerol as main carbon source and, several works have already demonstrated the possibility of obtaining a high variety of compounds from this alcohol (Amaral *et al.*, 2009; Chatzifragkou and Papanikolaou, 2012).

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is an intermediate organic acid of the tricarboxylic acids cycle. This compound is extensively used in numerous applications in food and pharmaceutical industry (Grewal and Kalra, 1995; Kamzolova *et al.*, 2008; Dhillon *et al.*, 2011a) and more recently, also in biomedical applications (Naeini *et al.*, 2010; Li *et al.*, 2012; Tran *et al.*,

2015). Citric acid is produced worldwide by *Aspergillus niger,* in submerged cultures from glucose syrups, sucrose or molasses. The constant increase in citric acid consumption and some problems in traditional production process (molasses treatment and environmental issues) leads to the need of exploring new microorganisms to be used, like yeasts species (Förster *et al.*, 2007a). The ability to use different carbon sources, to withstand high substrate concentrations and to tolerate the presence of metal ions in less refined substrates are some advantages of using yeasts in this production process.

Yarrowia lipolytica, a strictly aerobic and non-conventional yeast, is able to produce citric acid under nitrogen limited conditions with several carbon sources, including agro-industrial wastes. The production of citric acid from crude glycerol was described for the first time by Papanikolaou *et al.* (2002a). The production profile of this acid by *Y. lipolytica* varies as a result of many factors including the type of strain or the set of culture conditions. From these conditions, carbon and nitrogen source and respective concentrations, pH, temperature, dissolve oxygen available, salts concentration and other parameters directly influence citric acid production as long as the formation of other byproducts (Antonucci *et al.*, 2001).

The main goal of this thesis consists in the development of strategies to improve citric acid production by *Y. lipolytica* using crude glycerol from biodiesel industry. Considering the influence of culture conditions in citric acid production, the influence of carbon/nitrogen ratio, pH, oxygen mass transfer rate and salts concentration were assessed to established optimum culture conditions. Also different types of bioreactors were tested in this bioprocess: a traditional stirred tank bioreactor, a pressurized bioreactor and an airlift bioreactor. Finally, a set of mutagenic treatments were implemented in *Y. lipolytica,* to improve citric acid production and decrease the isocitric acid proportion produced simultaneously along the fermentation.

1.2 OUTLINE OF THE THESIS

The thesis was structured in eight chapters.

The current chapter (**Chapter 1**) presents the context, motivation and the research goals of this thesis. The structure and the scientific outputs of the thesis are also outlined.

In **Chapter 2** a review on the state of art of the biotechnological production of citric acid, its production by the yeast *Yarrowia lipolytica* is presented. Moreover, metabolic pathways involved in glycerol consumption and citric acid production and a brief overview about methods to increase the organic acid production are also addressed.

The different sections of **Experimental Results** are presented from **Chapter 3** to **Chapter 6**. In these chapters a brief *introduction*, *material and methods*, *results and discussion* and *conclusions* for the chapter topics are given.

In **Chapter 3** culture conditions were optimized for the maximization of citric acid production by two *Y. lipolytica* strains. The effect Carbon/Nitrogen (C/N) ratio, salts concentration, pH and oxygen mass transfer rate (OTR) on citric acid production by two strains of *Y. lipolytica* were studied applying an experimental design.

Chapter 4 presents the effect of k_La on citric acid production in a lab-scale stirred tank bioreactor (STR), by varying the stirring and the aeration rates. An empirical correlation for the prediction of k_La as a function of superficial gas velocity and power input of the aerated bioreactor with a correction in order to predict the effect in k_La of cells is also described.

In **Chapter 5** the use of two non-conventional bioreactors, pressurized and airlift on citric acid production by *Y. lipolytica* W29 is discussed. The effect of increase air pressure, using the pressurized bioreactor, and the aeration rate, in the airlift bioreactor, was studied.

Isolation of mutant strains after exposing *Y. lipolytica* W29 to mutagens and citric acid production by the mutants are described in **Chapter 6**.

Chapter 7 presents the overall conclusions obtained in this thesis and suggestions for future work.

Chapter 8 gathers all the references used in the elaboration of this work.

1.3 OUTPUTS OF THE THESIS

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

References

Ferreira, P., Lopes, M., Mota, M., Belo, I., Oxygen transfer rate and pH are major operating factors for citric acid production from glycerol by *Yarrowia lipolytica* W29 and CBS 2073, submitted to *Chemical Papers* (June 2015).

Ferreira, P., Lopes, M., Mota, M., Belo, I., Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol, submitted to *Biochemical Engineering Journal* (October 2015).

Ferreira, P., Lopes, M., Mota, M., Belo, I., Use of pressurized and airlift bioreactors for citric acid production by *Yarrowia lipolytica* from crude glycerol, submitted to *Process Biochemistry* (October 2015)

Some participation in conferences was used as means of learning new methodologies and to present the work developed along this thesis. The works presented at these events are listed below.

Poster presentation

- Ferreira, P., Mota, M., Belo, I., Production of citric acid from glycerol by *Yarrowia lipolytica* – optimization of culture conditions. ECAB2 - 2nd European Congress of Applied Biotechnology. The Hague, The Netherlands, April 21-25, 2013

- Ferreira, P., Mota, M., Belo, I., Citric acid production by *Yarrowia lipolytica* under increased air pressure. BioTech 2014 and 6th Czech-Swiss Symposium with Exhibition. No. P026-S, P-112, Prague, Czech Republic, June 11-14, 2014

- Gonçalves, A., Braga, A., **Ferreira, P.**, Belo, I., Immobilization of whole cells of *Yarrowia lipolytica* for citric acid production. CHEMPOR 2014 - Book of Extended Abstracts of the 12th International Chemical and Biological Engineering Conference. No. P-BE5, Porto, Portugal, Sep. 10-12, 2014.

- Ferreira, P., Mota, M., Belo, I., Citric acid production by *Yarrowia lipolytica* from crude glycerol: influence of oxygen mass transfer rate (OTR). Journal of Biotechnology, Volume: 208, Supplement: S, P-S48. Conference: European Biotechnology Congress Location: Bucharest, Romania, May 07-09, 2015.

2 LITERATURE REVIEW

Citric acid is an organic acid intermediate of tricarboxylic acid cycle widely used in food and pharmaceutical industries. This compound is traditionally produced by *Aspergillus niger* from molasses but the constant increase on annual consumption requires to implement new and cost effective alternatives for its production. *Yarrowia lipolytica* is a citric acid producer using crude glycerol from biodiesel industry that can be an interesting alternative to other processes of citric acid industry.

In this Chapter, the focus is the citric acid and its production by the yeast *Y. lipolytica*. A brief overview on metabolic pathways involved in glycerol consumption and citric acid production is presented, as well as strategies to improve organic acid production by this yeast are reported.

2.1 GLYCEROL

Glycerol, also known as 1,2,3-propanetiol, glycerin or glycerine, is a simple alcohol, nontoxic, colorless, odorless and viscous liquid with sweet-taste and hygroscopic properties. This alcohol is mainly used in chemical, textile, pharmaceutical, cosmetic, food, paint and automotive industries (Wang *et al.*, 2001). Glycerol can be produced by chemical synthesis, from petrochemical feedstock, by microbial fermentation, or as byproduct from soap manufacturing, alcoholic beverage and biodiesel industries (Wang *et al.*, 2001; Ardi *et al.*, 2015).

Biodiesel as renewable energy, became an important alternative to the fossil fuels in the transportation sector and created a new and expandable market (Johnson and Taconi, 2007). In Europe, according to European Biodiesel Board, biodiesel production capacity in 2014 was approximately 23 million tons, from which 2.5 % was Portugal's contribution (see http://www.ebb-eu.org). The biodiesel is produced from vegetable oils (triglycerides) through the transesterification with a monovalent alcohol, usually methanol (Figure 2.1) (Meher *et al.*, 2006). Glycerol is the main byproduct of this reaction, 1 kg of glycerol is produced per 10 kg of biodiesel.

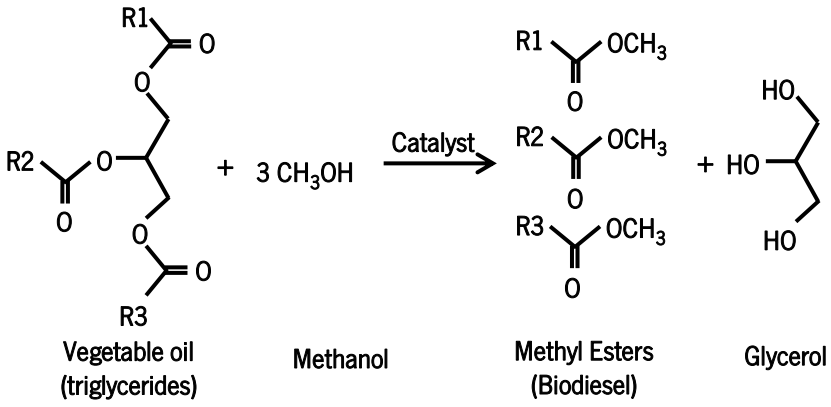

Figure 2.1 Transesterification reaction of vegetable oils for biodiesel production.

Considering the 2014 annual values of biodiesel production in Europe, 2 million tons of crude glycerol were produced solely by this industry and some countries are already treating crude glycerol as an industrial waste. Crude glycerol from the biodiesel presents typically 55 % to 90 % of glycerol being the remaining compounds unconverted alcohol, residual oil, salts, heavy metals and water (Johnson and Taconi, 2007; Amaral *et al.*, 2009). Due to its contaminants, the

use of this byproduct in the chemical and pharmaceutical industry (main application of glycerol which requires a high quality and purity grade) would demand a high cost purification process (Johnson and Taconi, 2007; Amaral *et al.*, 2009; Ardi *et al.*, 2015). Considering the high amounts of crude glycerol available and the cost of its purification, it is important to explore other processes that can use this mixture as it is produced. Biotechnological conversion of the glycerol in value-added products is one example of a possible application of this byproduct. In this field, recent works have focused in the production of value-added products by microbial fermentation of glycerol (Johnson and Taconi, 2007). Table 2.1 lists some compounds produced by microorganisms using crude glycerol as carbon source.

Table 2.1 Value-added products obtained by biotechnological conversion of crude glycerol by microorganisms.

Product	Microorganism	Reference
1,3-propanediol	*Colostridium butyricum*	(Papanikolaou *et al.*, 2008a; Wilkens *et al.*, 2012; Metsoviti *et al.*, 2012)
	Citrobacter freundii	(Anand and Saxena, 2012; Metsoviti *et al.*, 2012)
	Klebsiella pneumonia	(Jun *et al.*, 2010; Sattayasamitsathit *et al.*, 2011)
	Klebsiella oxytoca	(Metsoviti *et al.*, 2012)
meso-2,3-Butanediol	*Escherichia coli*	(Lee *et al.*, 2012)
2,3-butanediol	*Klebsiella pneumonia*	(Sattayasamitsathit *et al.*, 2011)
	Clostridium butyricum *Citrobacter freundii* *Enterobacter aerogenes* *Klebsiella oxytoca*	(Metsoviti *et al.*, 2012)
poly(3-hydroxybutyrate) (PHB)	*Paracoccus denitrificans* *Cupriavidus necator*	(Mothes *et al.*, 2007)
Ethanol	*Klebsiella oxytoca*	(Metsoviti *et al.*, 2012)
	Pachysolen tannophilus	(Liu *et al.*, 2012)
	Kluyvera cryocrescens	(Choi *et al.*, 2011)
Erythritol	*Yarrowia lipolytica*	(Tomaszewska *et al.*, 2012; 2014)
Mannitol	*Yarrowia lipolytica*	(Tomaszewska *et al.*, 2012)

(continue)

Table 2.1 Value-added products obtained by biotechnological conversion of crude glycerol by microorganisms. (continuation)

Product	Microorganism	Reference
Propionic acid	*Propionibacterium freudenreichii* subspp. *shermanii*	(Ruhal and Choudhury, 2012)
Lactic acid	*Propionibacterium freudenreichii* subspp. *shermanii*	(Ruhal and Choudhury, 2012)
Citric acid	*Yarrowia lipolytica*	(Papanikolaou *et al.*, 2008a)
Food pigments	*Blakeslea trispora*	(Mantzouridou *et al.*, 2008)
	Rhodotorula glutinis	(Saenge *et al.*, 2011)
Trehalose	*Propionibacterium freudenreichii* subspp. *shermanii*	(Ruhal and Choudhury, 2012)
Hydrogen	*Rhodopseudomonas palustris*	(Sabourin-Provost and Hallenbeck, 2009)
Vitamin B_{12}	*Propionibacterium freudenreichii* subspp. *shermanii*	(Kośmider *et al.*, 2012)
Lipids	*Yarrowia lipolytica*	(Papanikolaou *et al.*, 2008a; Poli *et al.*, 2014)
	Rhodotorula glutinis	(Saenge *et al.*, 2011)
Phytase	*Pichia pastoris*	(Tang *et al.*, 2009)
Lipase	*Staphylococcus caseolyticus*	(Volpato *et al.*, 2008)

2.2 CITRIC ACID

Citric acid or 2-hydroxy-propane1,2,3-tricarboxylic acid (Figure 2.2) is an weak organic acid intermediate of the tricarboxylic acids (TCA) cycle found in all citric fruits. This tricarboxylic acid is accepted as GRAS (Generally Recognized As Safe) and approved by the Joint FAO/WHO Expert Committee on Food Additives (Dhillon *et al.*, 2011a). In its pure form, this compound is colorless, soluble in water and solid at room temperature. Citric acid presents, at 20 °C, three pK_a values at pH 3.1, 4.7 and 6.4, characteristic that makes it a fantastic buffer (Show *et al.*, 2015). This acid also can form several metallic salts reacting with copper, iron magnesium, manganese and calcium. All this characteristics make citric acid a very attractive compound to several industries being its main applications as a buffer, pH adjustment, chelating and derivatization agent (Mattey

and Kristiansen, 1999; Dhillon *et al.*, 2011a). For example, citric acid is used as a food ingredient in the production of jams, cheese, ice creams, fruit/vegetable juices wines and ciders. It is also applicable in pharmaceutical industry as an ingredient of buffer syrups, anticoagulant, antioxidant, in cosmetic industry, and manufacture of detergents (Grewal and Kalra, 1995; Kamzolova *et al.*, 2008; Dhillon *et al.*, 2011a). Some recent studies reveal that citric acid can be used as biopolymer for tissue engineering culturing cells, drug delivery, and other biomedical applications (Naeini *et al.*, 2010; Li *et al.*, 2012; Tran *et al.*, 2015). The vast versatility of citric acid in many industries and the novel applications justify its high demand, which increases about 4 % each year (Show *et al.*, 2015). The worldwide production of citric acid was in 2007 around 1.7 million tons (Dhillon *et al.*, 2011a).

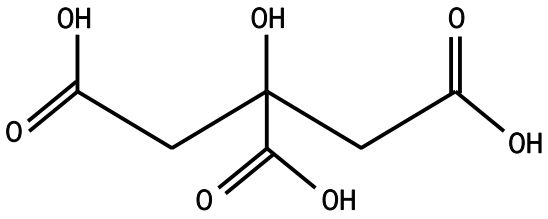

Figure 2.2 Chemical structure of citric acid.

Citric acid was isolated for the first time from lemon juice by a Swedish chemical named Carl Scheele in 1784. In 1826, citric acid extracted from Italian lemons started to be commercialized in England. This was the only commercial source of this acid for many years. The chemical synthesis of citric acid was developed, later, from glycerol or dichloroacetone. Other synthetic routes were published after, but none was considered competitive or suitable, mainly for economic reasons (Mattey and Kristiansen, 1999; Papagianni, 2007).

The production by microorganisms was the last route to be explored. In 1880, was described that the genus *Citromyces* (now renamed as *Penicillum*) was able to accumulate citric acid. This work triggered several other studies towards the search of other microorganisms capable of producing citric acid. Numerous fungi were described with this capability, but was Currie in 1917 who described that some *Aspergillus niger* strains growing in high sugar concentration and low initial pH could secrete high amounts of citric acid. This work aroused interest of some pharmaceutical companies that later implemented this process and started to

produce citric acid by a bioprocess mechanism - fermentation (Papagianni, 2007). Many studies describing other fungi, bacteria and yeast as citric acid producers capable to produce and accumulate citric acid in high amounts followed soon after the implementation of fermentation by *A. niger*, (Table 2.2). However, most of citric acid continues to be manufactured by *A. niger* in submerged fermentation from molasses (sucrose) and starch hydrolysates (glucose). Submerged fermentation is the most used technology, although citric acid can also be produced by surface fermentation (its first industrial manufacture process) (Roukas and Kotzekidou, 1986; Darouneh *et al.*, 2009) and solid-state fermentation (Koji process) (Shojaosadati and Babaeipour, 2002; Yadegary *et al.*, 2013).

Molasses are a byproduct from the sugar industry (cane and beet), produced in the final stage of crystallization in the sugar production process. This byproduct is extensively used as carbon source in citric acid production by *A. niger,* due to its low cost compared with more refined sugar sources. Beside molasses, some agro-industrial wastes have been studied as an alternative low-cost carbon source. Apple pomace (Hang and Woodams, 1984; Dhillon *et al.*, 2011b),corn cobs (Hang and Woodams, 1998), carob pod (Roukas, 1999), kumara (Lu and Brooks, 1995; Lu *et al.*, 1997), kiwi fruit peel (Hang *et al.*, 1987), pineapple waste (Tran and Mitchell, 1995; Kumar *et al.*, 2003), orange waste (Torrado *et al.*, 2011), banana extract (Sassi *et al.*, 1991; Karthikeyan and Sivakumar, 2010), pumpkin (Majumder *et al.*, 2010), brewery spent grain (Hang *et al.*, 1975; Aregbesola and Omafivbe, 2014) and jackfruit wastes (Angumeenal and Venkappayya, 2005) were successfully used as substrates for citric acid formation.

The production of citric acid by *A. niger* from molasses is a well-established process but still presents a few drawbacks, such as: (a) the use of molasses from sugar manufactory requires purification, increasing the process costs and (b) environmental issues related with the wastes generated during the production (Förster *et al.*, 2007a). The issues related to this process and the constant increase of citric acid demand has generated urgency towards the need of exploring new species for citric acid production. Some yeasts can produced and accumulate citric acid and be an alternative to produce this organic acid production process (Soccol *et al.*, 2006; Dhillon *et al.*, 2011a). Yeast species as citric acid producers present several advantages, such as: (a) capability of using a wide range of substrates, (b) tolerance to higher substrate concentration, (c) less sensitivity to metal ions, allowing the use of crude carbon sources without any treatment, (d)

greater conversion rate and (e) better process control due to unicellular nature of yeast (Grewal and Kalra, 1995; Karasu-Yalcin, 2012).

Table 2.2 Species and genera able to produce citric acid (Grewal and Kalra, 1995; Mattey and Kristiansen, 1999; Soccol *et al.*, 2006; Dhillon *et al.*, 2011a).

Filamentous fungi	Yeasts	Bacteria
Citromyces (now *Penicillium*)	*Candida oleophils*	*Arthrobacter paraffinens*
Aspergillus niger	*Candida guilliermondii*	*Bacillus licheniformis*
Aspergillus awamori	*Candida catenula*	*Corynebacterium* sp.
Aspergillus nidulans	*Candida tropicalis*	
Aspergillus fonsecaeus	*Candida citroformans*	
Aspergillus luchensis,	*Candida intermediate*	
Aspergillus phoenicus	*Candida parapsilosis*	
Aspergillus wentii,	*Hansenula anamola*	
Aspergillus saitoi,	*Yarrowia lipolytica*	
Aspergillus flavus,	*Pichia* sp.	
Aspergillus aculeatus	*Debaromyces* sp.	
Aspergillus carbonarius	*Torula* sp.	
Aspergillus foetidus	*Torulopsis* sp.	
Aspergillus lanosius	*Kloekera* sp.	
Mucor piriformi	*Saccharomyces* sp.	
Penicillium janthinellum	*Zygosaccharomycea* sp.	
Penicillium restrictum		
Trichoderma viride		
Ustulina vulgaris		
Absidia sp.		
Acremonium sp.		
Botrytis sp.		
Eupenicillium sp.		
Talaromyces sp.		
Aschochyta sp.		

2.3 *YARROWIA LIPOLYTICA*

Yarrowia lipolytica, an eukaryotic, strictly aerobic microorganism, belongs to the Fungi Kingdom, Ascomycete class, Hemiascomycetes subclass, Saccharomycetales order and Dipodascacea family. This yeast was firstly named as *Candida lipolytica*, since no sexual state has been described (Barth and Gaillardin, 1997). Later, Wickerham *et al.*, (1970) described the perfect form of *C. lipolytica* that was renamed as *Endomycopsis lipolytica* and then as *Saccharomycopsis lipolytica* (Yarrow, 1972). In 1980 van der Walt and von Arx proposed the name of *Yarrowia lipolytica*, "*Yarrowia*" in honor to David Yarrow, for the new genus identified and "*lipolytica*" for its capacity of hydrolyze lipids (van der Walt and von Arx, 1980).

Y. lipolytica is one of the most well studied "non-conventional" yeast. This term differentiate *Y. lipolytica* and other yeast from the more commonly used and well-studied yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, regarding its phylogenetic evolution, physiology, genetics and molecular biology (Barth and Gaillardin, 1997).

Natural dimorphic yeast, *Y. lipolytica* is capable of growing in two different morphological forms (Figure 2.3), as single oval form or as filamentous hyphae. The dimorphism was been described as consequence of a reaction mechanism to adverse conditions, being reversible between each other (Kawasse *et al.*, 2003). The cell shape is influenced by environmental conditions (pH, dissolved oxygen concentration in the medium, carbon and nitrogen sources, some minerals, etc.) and by the genetic background of the strain (Barth and Gaillardin, 1997; Pérez-Campo and Domínguez, 2001; Ruiz-Herrera and Sentandreu, 2002; Kawasse *et al.*, 2003).

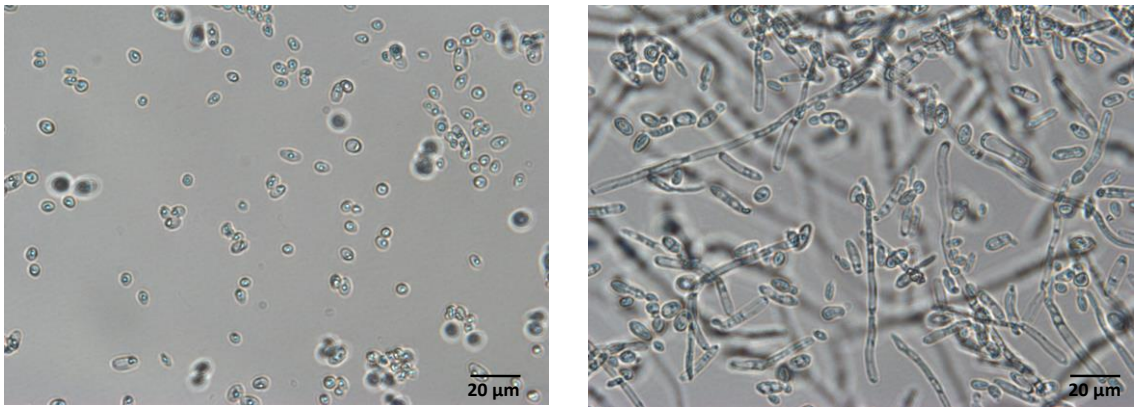

Figure 2.3 Cell morphology of *Y. lipolytica* W29: single oval form (left) and filamentous hyphae (right) (magnification 400x).

Y. lipolytica is a non-pathogenic yeast (Holzschu *et al.*, 1979), classified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration, USA). This yeast can be isolated from food rich in fat and proteins, like dairy products (cheese, yogurts) (Roostita and Fleet, 1996; Suzzi *et al.*, 2001; Vasdinyei and Deák, 2003), chilled products (sausages) (Guerzoni *et al.*, 1993; Gardini *et al.*, 2001), from soil, sewage and oil-polluted environments (Kim *et al.*, 1999; Schmitz *et al.*, 2000; Thevenieau *et al.*, 2009; Mafakher *et al.*, 2010). This wide range of habitats reflects the versatility of this yeast metabolism. *Y. lipolytica* is able to use hydrophobic substrates like n-alkanes (Fukuda, 2013), n-paraffins (Crolla and Kennedy, 2004), oils and fatty acids (Papanikolaou and Aggelis, 2003a), which makes it an interesting microorganism for bioremediation processes (Bankar *et al.*, 2009). In addition to hydrophobic substrates, other carbons sources, like alcohols such as ethanol (Il'chenko *et al.*, 2002) and glycerol (Papanikolaou *et al.*, 2002b), organic acids (Rodrigues and Pais, 2000) and sugars (glucose, fructose) are used by *Y. lipolytica* (Barth and Gaillardin, 1997; Coelho *et al.*, 2010). Moreover, this species has been proved to be very efficient using carbon sources from agro-industrial wastes like olive mill waste water (Gonçalves *et al.*, 2009), vegetable oils residues (Louhasakul and Cheirsilp, 2013; Saygün *et al.*, 2014), animal fat (Kamzolova *et al.*, 2005), and crude glycerol from biodiesel industry (Rywińska *et al.*, 2013).

The high interest on this yeast started with the discovery of its capacity of using hydrophobic substrates, like n-alkanes, fatty acids and oils and produce single-cell protein (SCP). The ability of secrete high amounts of protein triggered the development of molecular biology and genetic tools, increasing the interest of using this yeast for academic and industrial purposes (Fickers, *et al.*, 2005; Nicaud, 2012). Currently, *Y. lipolytica* is used as a model to study dimorphism (Domínguez *et al.*, 2000), degradation of hydrophobic substrate (Fickers, *et al.*, 2005), protein secretion (Beckerich *et al.*, 1998; Nicaud *et al.*, 2002; Madzak *et al.*, 2004), peroxisome biogenesis (Titorenko *et al.*, 2000), lipid body biogenesis (Beopoulos *et al.*, 2008; Beopoulos, *et al.*, 2009a; 2009b), and analysis of mitochondrial complex I (Kerscher *et al.*, 2002). Along with the proteins secretion and lipid accumulation, *Y. lipolytica* can also produce other value-added products, such as aromas (Romero-Guido *et al.*, 2011), microbial lipids (Beopoulos *et al.*, 2009a), organic acids (Otto *et al.*, 2013), biosurfactants (Amaral *et al.*, 2006) and enzymes like lipases (Fickers *et al.*, 2011), phosphatases, esterases, proteases and RNases, (Barth and Gaillardin, 1997).

2.4 CITRIC ACID: PRODUCTION BY *YARROWIA LIPOLYTICA*

Y. lipolytica, as citric acid producer, has received much attention due to it greater resistance to high substrate concentrations, tolerance to metal ions presence and wide range of possible substrates, simple controlled process and waste minimization (Otto *et al.*, 2013). Growing under nutrient-limited conditions, *Y. lipolytica* is able to synthesize citric acid from a high variety of substrates. These sources include sugars, n-hydrocarbons, alcohols and plant oils. In table 2.3 are summarized some studies of citric acid production from different carbon sources by *Y. lipolytica* strains.

Table 2.3 Citric acid production by different *Y. lipolytica* strains using different carbon sources.

Strain	Fermentation type	Substrate	Initial substrate concentration	Maximum citric acid concentration	Reference
Y. lipolytica A101	Repeated-batch using immobilized cells (Flask)	Glucose	60 $g \cdot L^{-1}$	1.0 to 19.4 $g \cdot L^{-1}$	(Kautola *et al.*, 1991)
Y. lipolytica A101	Batch (flask)	n-paraffins	50 $g \cdot L^{-1}$	69.3 $g \cdot L^{-1}$ (Citric +isocitric acid)	(Wojtatowicz *et al.*, 1993)
Candida lipolytica Y 1095	Batch	Glucose	50 - 150 $g \cdot L^{-1}$	13.6 – 78.8 $g \cdot L^{-1}$	(Rane and Sims, 1993)
Mutants of Y. lipolytica VKM Y-2373	Repeated-batch	Ethanol	Maintained at maximum of 1.2 $g \cdot L^{-1}$	105.4 $g \cdot L^{-1}$	(Arzumanov *et al.*, 2000)
C. lipolytica NRRL-Y-1095	Batch (flask)	n-paraffins	157 $mL \cdot L^{-1}$	9.8 $g \cdot L^{-1}$	(Crolla and Kennedy, 2001)
Y. lipolytica N1	Continuous	Ethanol	0.01-1 $g \cdot L^{-1}$	14.2 - 19.2 $g \cdot L^{-1}$	(Finogenova *et al.*, 2002)
Y. lipolytica N1	Continuous	Ethanol	5.6 % (v/v)	120.0 $g \cdot L^{-1}$	(Kamzolova *et al.*, 2003)
Y. lipolytica UOFS Y-1701	Batch (flask)	Sunflower oil + acetate	30 $g \cdot L^{-1}$ + 10 $g \cdot L^{-1}$	18.7 $g \cdot L^{-1}$	(Venter *et al.*, 2004)
C. lipolytica NRRL-Y-1095	Fed-Batch	n-paraffins	157 $mL \cdot L^{-1}$	42.0 $g \cdot L^{-1}$	(Crolla and Kennedy, 2004)
Y. lipolytica 187/1	Repeated-batch	Rapeseed oil	Maintained above 5 $g \cdot L^{-1}$	135.0 $g \cdot L^{-1}$	(Kamzolova *et al.*, 2005)

(continue)

Table 2.3 Citric acid production by different *Y. lipolytica* strains using different carbon sources. (continuation)

Strain	Fermentation type	Substrate	Initial substrate concentration	Maximum citric acid concentration	Reference
Y. lipolytica NRR YB-423	Batch (flask)	Glycerol	40 g·L^{-1}	21.8 g·L^{-1}	(Levinson *et al.*, 2007)
Y. lipolytica H222-S4(p67ILC1)T5	Fed-Batch	Sucrose	Total added 170 g·L^{-1}	140.0 g·L^{-1}	(Förster *et al.*, 2007a)
Y. lipolytica VKM Y-2373	Batch (flask)	Glucose	30 g·L^{-1}	17.6 g·L^{-1}	(Finogenova *et al.*, 2008)
Mutants of Y. lipolytica VKM Y-2373				4.3 to 19.9 g·L^{-1}	
Y. lipolytica VKM Y-2373	Repeated-batch	Sunflower Oil	20 g·L^{-1} (add when concentration was <5 g·L^{-1})	68.0 g·L^{-1}	(Kamzolova *et al.*, 2008)
Y. lipolytica N1				150.0 g·L^{-1}	
Y. lipolytica NBRC 1658	Batch	Glucose	150 g·L^{-1}	34.2 g·L^{-1}	(Karasu-Yalcin *et al.*, 2009b)
		Fructose		33.7 g·L^{-1}	
Y. lipolytica 57		Glucose	150 g·L^{-1}	44.3 g·L^{-1}	
		Fructose	200 g·L^{-1}	65.1 g·L^{-1}	
Y. lipolytica AWG7	Fed-batch	Glycerol	80 - 100 g·L^{-1}	139.0 g·L^{-1}	(Rymowicz *et al.*, 2009)
Y. lipolytica Wratislavia K1				89.0 g·L^{-1}	
Y. lipolytica 57	Batch	Glycerol	160 g·L^{-1}	32.8 g·L^{-1}	(Karasu-Yalcin *et al.*, 2009a)
		Mannitol	120 g·L^{-1}	21.7 g·L^{-1}	
Y. lipolytica NBRC 1658		Glycerol	120 g·L^{-1}	21.5 g·L^{-1}	
		Mannitol		20.3 g·L^{-1}	
Y. lipolytica (several strains)	Batch (flask)	Glucose	30 g·L^{-1}	1.9 to 18.0 g·L^{-1}	(Papanikolaou *et al.*, 2009)
			60 g·L^{-1}	43.6 to 59.8 g·L^{-1}	
Y. lipolytica AWG7	Repeated-batch	Glycerol	Total 200 g·L^{-1}	154.0 g·L^{-1}	(Rywińska and Rymowicz, 2010)

(continue)

Table 2.3 Citric acid production by different *Y. lipolytica* strains using different carbon sources. (continuation)

Strain	Fermentation type	Substrate	Initial substrate concentration	Maximum citric acid concentration	Reference
Y. lipolytica NBRC 1658	Batch (flask)	Glucose	100 g·L^{-1}	30.0 g·L^{-1}	(Karasu-Yalcin et al., 2010)
Y. lipolytica 57				38.7 g·L^{-1}	
Y. lipolytica A-101	Batch	Glycerol	150 g·L^{-1}	66.0 g·L^{-1}	(Rywińska *et al.*, 2010)
		Glucose		69.3 g·L^{-1}	
Y. lipolytica Wratislavia 1.31		Glycerol		82.0 g·L^{-1}	
		Glucose		76.4 g·L^{-1}	
Y. lipolytica Wratislavia AWG7		Glycerol		82.9 g·L^{-1}	
		Glucose		78.5 g·L^{-1}	
Y. lipolytica Wratislavia K1		Glycerol		53.3 g·L^{-1}	
		Glucose		49.5 g·L^{-1}	
Y. lipolytica ACA-DC-50109	Repeated batch	Glycerol	104.9 g·L^{-1}	40 g·L^{-1}	(Makri *et al.*, 2010)
Y. lipolytica M1	Batch (flask)	Glucose	100 g·L^{-1}	27.0 g·L^{-1}	(Mafakher *et al.*, 2010)
Y. lipolytica M2				8.0 g·L^{-1}	
Y. lipolytica H222	Repeated batch	Glucose	150 g·L^{-1}	98.0 g·L^{-1}	(Moeller *et al.*, 2011)
Y. lipolytica NG40/UV7	Batch	Rapeseed oil	140 g·L^{-1}	175.0 g·L^{-1}	(Kamzolova *et al.*, 2011a)
Y. lipolytica A-101-B56-5	Batch	Glucose	100 g·L^{-1}	46.2 g·L^{-1}	(Lazar *et al.*, 2011)
		Glucose + Fructose	50 g·L^{-1} + 50 g·L^{-1}	49.6 g·L^{-1}	
		Sucrose	100 g·L^{-1}	45.0 g·L^{-1}	
		Glycerol	100 g·L^{-1}	57.1 g·L^{-1}	
Y. lipolytica DSM 3286	Batch (flask)	Glucose	100 g·L^{-1}	75.0 g·L^{-1}	(Mirbagheri *et al.*, 2011)
Y. lipolytica M7				85.0 g·L^{-1}	

(continue)

Table 2.3 Citric acid production by different *Y. lipolytica* strains using different carbon sources. (continuation)

Strain	Fermentation type	Substrate	Initial substrate concentration	Maximum citric acid concentration	Reference
Y. lipolytica AWG7	Continuous	Glycerol	150 g·L^{-1}	97.8 g·L^{-1}	(Rywińska *et al.*, 2011)
Y. lipolytica N15	Batch	Glycerol	170 g·L^{-1}	98.0 g·L^{-1}	(Kamzolova *et al.*, 2011b)
Y. lipolytica Wratislavia 1.31	Batch	Glycerol	150 g·L^{-1}	NA	(Rywińska *et al.*, 2012)
Y. lipolytica Wratislavia AWG7				NA	
Y. lipolytica SWJ-1b - transformant 30	Batch	Jerusalem artichoke tubercles	Total sugar 84.3 g·L^{-1}	68.3 g·L^{-1}	(Wang *et al.*, 2013)
Yarrowia lipolytica H222-S4(p67ICL1)T5	Fed-batch	Sucrose	120 g·L^{-1}	114.5 g·L^{-1}	(Moeller *et al.*, 2013)
Y. lipolytica YB423-12	Batch (flask)	Borage oil	10 g·L^{-1}	5.3 g·L^{-1}	(Saygün *et al.*, 2014)
Y. lipolytica Wratislavia 1.31	Batch	Glycerol	150 g·L^{-1}	76.0 g·L^{-1}	(Tomaszewska *et al.*, 2014)
Y. lipolytica Wratislavia AWG7				85.7 g·L^{-1}	
Y. lipolytica Wratislavia K1				65.0 g·L^{-1}	
Y. lipolytica TEM YL3	Batch (flasks)	Glucose	100 g·L^{-1}	33.3 g·L^{-1}	(Çelik *et al.*, 2014)
		Glycerol		35.6 g·L^{-1}	
		Sunflower oil		66.2 g·L^{-1}	
Y. lipolytica TEM YL20		Glucose	100 g·L^{-1}	36.3 g·L^{-1}	
		Glycerol		37.5 g·L^{-1}	
		Sunflower oil		50.7 g·L^{-1}	
Yarrowia lipolytica K-168	Batch (flasks)	Carrot juice	190.3 g·L^{-1} (total sugar)	80.5 g·L^{-1}	(Urak *et al.*, 2015)

NA - not available

The tolerance of *Y. lipolytica* to metal ions allows the use of less refined carbon sources in citric acid production, like byproducts and wastes of other industries. Studies using agro-industrial wastes and byproducts as a low cost substrate in acid citric production by *Y. lipolytica* are summarized in table 2.4.

Table 2.4 Studies using agro-industrial wastes and byproducts in citric acid production by different *Y. lipolytica* strains

Y. *lipolytica* strain	Fermentation type	Substrate	Initial substrate concentration	Citric acid concentration	Reference
A-101-1.14	Batch	Glucose Hydrol	40 % (v/v)	100 $g \cdot L^{-1}$	(Wojtatowicz *et al.*, 1991)
A-101-1.22	Batch	Beet molasses	200 $g \cdot L^{-1}$	58.2 $g \cdot L^{-1}$	(Żarowska *et al.*, 2001)
A-101-1.31				46.9 $g \cdot L^{-1}$	
NCIM 35.89	Solid state	Pineapple waste	NA	202.35 $g \cdot kg^{-1}$	(Imandi *et al.*, 2008)
ACA-DC50109	Batch	Glucose on Olive mill wastewater based medium	65 $g \cdot L^{-1}$ + 30 % (v/v)	28.9 $g \cdot L^{-1}$	(Papanikolaou *et al.*, 2008b)
W29 (ATCC 20460	Batch (flask)	Glucose on Olive mill wastewater medium (phenolic compounds)	30 $g \cdot L^{-1}$ + 0 - 1.50 $g \cdot L^{-1}$	0.9 - 18.5 $g \cdot L^{-1}$	(Sarris *et al.*, 2011)
ACA-YC 5028				7.4 - 9.4 $g \cdot L^{-1}$	
ACA-YC 5033				13.8 - 18.9 $g \cdot L^{-1}$	
K-168	Batch (flask)	Carrot juice + glucose	30 % (v/v) + 140 $g \cdot L^{-1}$	62.6 $g \cdot L^{-1}$	(Karasu-Yalcin, 2012)
		Celery + glucose	100 % (v/v) + 100 $g \cdot L^{-1}$	15.78 $g \cdot L^{-1}$	
SWJ-1b	Batch	Cooking oil	80 $g \cdot L^{-1}$	31.7 $g \cdot L^{-1}$	(Liu *et al.*, 2014)

NA - not available

In 2002, the use of crude glycerol from biodiesel industry as a carbon source for citric acid production by *Y. lipolytica* was reported (Papanikolaou *et al.*, 2002a). Since then, other authors have been studied and optimizing citric acid production by several strains of *Y. lipolytica* using this low cost substrate (Table 2.5).

Table 2.5 Citric acid production by several strains of *Y. lipolytica* from crude glycerol.

Yarrowia lipolytica strain	Fermentation type	Initial substrate concentration	Maximum citric acid concentration	Reference
LGAM S(7)1	Batch (flask)	120 g·L^{-1}	35.0 g·L^{-1}	(Papanikolaou *et al.*, 2002a)
1.31	Batch	200 g·L^{-1}	124.5 g·L^{-1}	(Rymowicz *et al.*, 2006)
AWG7			88.1 g·L^{-1}	
K1			75.7 g·L^{-1}	
Wratislavia K1	Fed-batch	150 g·L^{-1}	110.0 g·L^{-1}	(Rymowicz *et al.*, 2008)
AWG7	Fed-batch	80 - 100 g·L^{-1}	131.5 g·L^{-1}	(Rywińska et al., 2009)
Wratislavia K1			86.8 g·L^{-1}	
ACA-YC 5033	Batch	120 g·L^{-1}	50.1 g·L^{-1}	(André *et al.*, 2009)
A-101	Batch	150 g·L^{-1}	66.8 g·L^{-1}	(Rywińska *et al.*, 2010)
Wratislavia 1.31			63.0 g·L^{-1}	
Wratislavia AWG7			62.0 g·L^{-1}	
Wratislavia K1			36.8 g·L^{-1}	
A-101-1.22	Batch	125 g·L^{-1}	112.0 g·L^{-1}	(Rymowicz *et al.*, 2010)
	Repeated-batch	250 g·L^{-1}	124.2 g·L^{-1}	
	Cell recycle	187.5 g·L^{-1}	107.0 g·L^{-1}	
AWG7	Continuous	250 g·L^{-1}	116.0 g·L^{-1}	(Rywińska and Rymowicz, 2011)
Wratislavia 1.31			78.0 g·L^{-1}	
N15	Batch	100 g·L^{-1}	71.0 g·L^{-1}	(Kamzolova *et al.*, 2011b)
NG40/UV7	Repeated-batch	20 g·L^{-1}	112.0 g·L^{-1}	(Morgunov *et al.*, 2013)
Wratislavia K1	Batch	175 g·L^{-1}	40.6 g·L^{-1}	(Tomaszewska *et al.*, 2014)
VKM Y-2373	Repeated-batch	20 g·L^{-1}	82.1 g·L^{-1}	(Kamzolova *et al.*, 2015)
VKM Y-2373V	Fed-batch	20 g·L^{-1}	67.7 g·L^{-1}	(Morgunov and Kamzolova, 2015)
NG40/UV7			122.2 g·L^{-1}	

During aerobic catabolism (Figure 2.4), glycerol crosses the cell membrane by facilitated diffusion and less frequently by active transport (Fakas *et al.*, 2009). Inside the cell, glycerol is phosphorylated into 3-P-glycerol by glycerol kinase, and then oxidized by a NAD-linked dehydrogenase to 3-P-dihydroxyacetone, which is converted by a triose phosphate isomerase into 3-P-glyceraldehyde. 3-P-glyceraldehyde can be used in gluconeogenesis, to produce hexoses and some sugar alcohols, like erythritol and mannitol or enters in the glycolytic pathway. In glycolytic pathway, 3-P-glyceraldehyde is converted into pyruvic acid which is subsequently transported to the mitochondrion where it undergoes an oxidative decarboxylation catalyzed by pyruvate dehydrogenase. This leads to the formation of acetyl-CoA molecule and the reduction of NADH. Acetyl-CoA is a precursor for a variety of metabolic compounds like, free fatty acids and organic acids, through TCA cycle (Flores *et al.*, 2000; Fakas *et al.*, 2009).

In the TCA cycle, acetyl-CoA condensates with oxaloacetate to form citrate by citrate synthase. In the next step, aconitase isomerizes citrate into isocitrate, which is subsequently oxidized by isocitrate dehydrogenase into α-ketoglutarate, releasing a CO_2 molecule. An oxidative decarboxylation reaction catalyzed by α-ketoglutarate dehydrogenase converts α-ketoglutarate into succinyl-CoA which is converted to succinate by succinily-CoA synthetase, then succinate is oxidized by succinate dehydrogenase into fumarate that is converted to malate by fumarase. Finally, malate is converted into oxaloacetate by an oxidation reaction catalyzed by malate dehydrogenase (Flores *et al.*, 2000; Gonçalves *et al.*, 2014).

Glyoxylate cycle is an anaplerotic pathway, source of intermediates of TCA cycle when necessary. This cycle has two important enzymes, isocitrate lyase and malate synthase and takes place in peroxissome. Isocitrate lyase catalyzes the cleavage of isocitrate into succinate and glyoxylate and malate synthase catalyzes the condensation of glyoxylate with a molecule of acetyl CoA into malate. The succinate is then transported to the mitochondrion and integrates the TCA cycle, while malate is converted into oxaloacetate, closing the cycle (Flores *et al.*, 2000).

The TCA cycle can be hampered by the exhaustion of nitrogen in the cell, leading to an accumulation of citric acid. In more detail (Figure 2.4), when the source of nitrogen ends there is a decrease of the intracellular AMP pool, due to its cleavage by AMP-desaminase. Thus NAD^+-(and also $NADP^+$-) isocitrate dehydrogenase activity decreases (enzyme catalyzed the formation of isocitrate into α-ketoglutarate) since it is allosterically activated by intracellular AMP. As result, there is an increase in the intracellular pool of citrate and isocitrate, which is subsequently

favored towards the accumulation of citrate through the action of aconitase. At a certain point, where the concentration of citrate reaches a critical value, this acid is excreted into the extracellular space (Morgunov *et al.*, 2004; Papanikolaou and Aggelis, 2009).

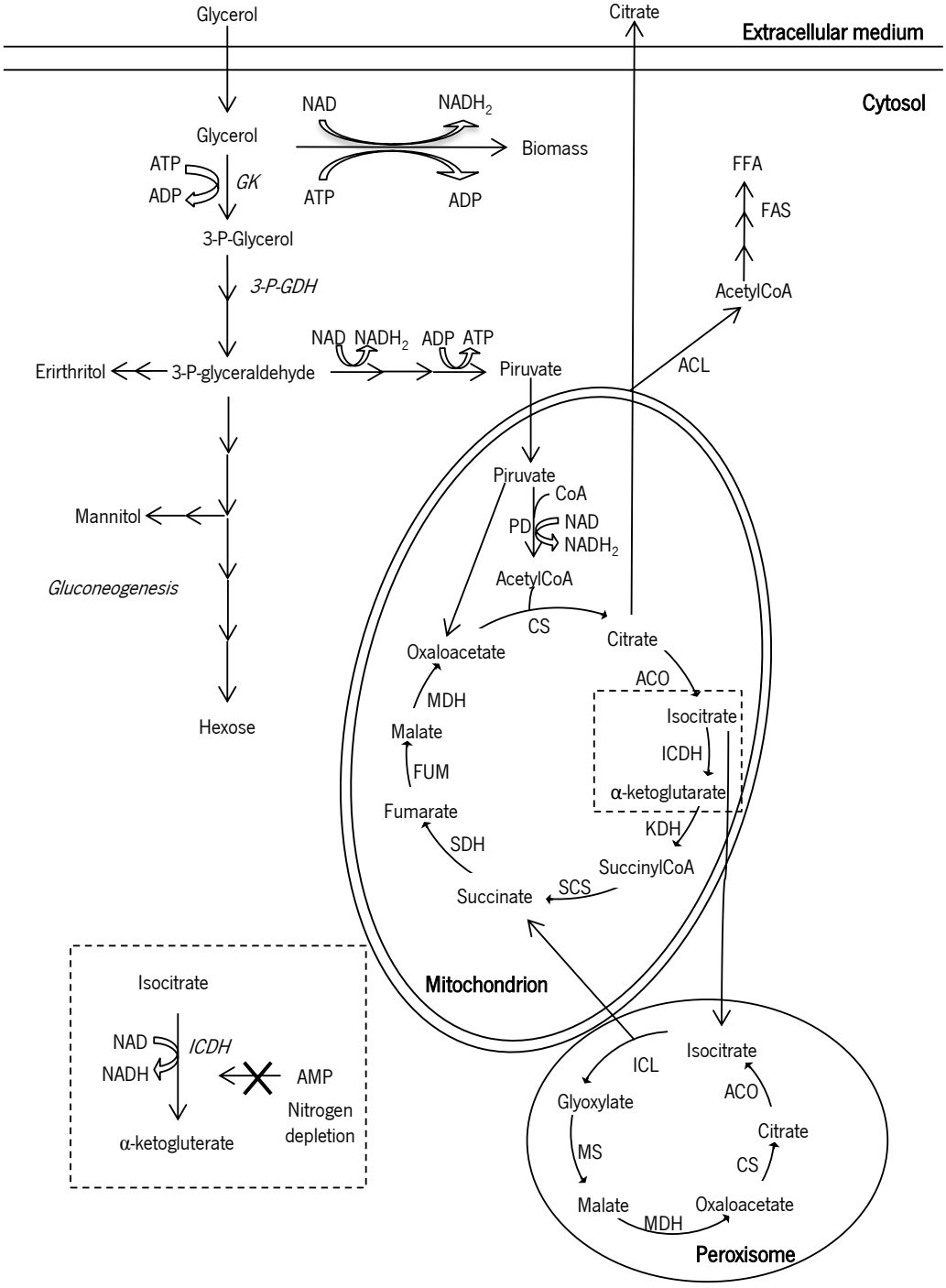

Figure 2.4 Pathways involved in the glycerol catabolism by *Y. lipolytica*. Abbreviations: GK - glycerol kinase; 3-P-GDH - 3-P-glyceroldehydrogenase; PD - pyruvate dehydrogenase; CS - citrate synthase; ACO – Aconitate; ICDH - iso-citrate dehydrogenase; KDH - α-ketoglutarate dehydrogenase ; SCS – succinily-CoA synthetase; SDH – succinate dehydrogenase; FUM – Fumarase; MDH - malate dehydrogenase; ICL - isocitrate lyase; MS - malate synthase; ACL – ATP-Citrate lyase; FAS – Fatty acid synthetase; FFA – Free fatty acids.

Y. lipolytica has great potential to be used in the production of citric acid, however the simultaneous production isocitric acid represents a major drawback that limits the use of this yeast at industrial scale. Isocitric acid has a lower buffer capacity and chelating ability than citric acid. Even a small amount of around 5 % can affect crystallization of citric acid, and remains a critical problem during purification process (Holz *et al.*, 2009). The isocitric/citric acid ratio depends mainly on the strains and the carbon source used, but culture conditions also affect this parameter. To reduce this ratio an improve citric acid production, several approaches can be investigated as: a) culture conditions optimization b) strain improvement by mutagenesis (Finogenova *et al.*, 2008; Rywińska *et al.*, 2010) or c) by genetic transformation (Förster *et al.*, 2007b; Holz *et al.*, 2011; Liu *et al.*, 2013).

2.5 IMPROVEMENT STRATEGIES OF CITRIC ACID PRODUCTION BY *YARROWIA LIPOLYTICA*

The production profile of citric acid by *Y. lipolytica* is strongly affected by the strain used but also by modifying the culture conditions. Different nitrogen sources and concentrations, carbon sources and concentrations, pH, temperature, oxygen, salts and other parameters can directly influence citric acid production and the formation of byproducts (Antonucci *et al.*, 2001).

2.5.1 Effect of culture conditions

The production and accumulation of citric acid by *Y. lipolytica* occurs under limited growth conditions and a carbon source excess. Citric acid accumulation starts at the stationary phase, when the growth is restricted by specific nutrient limitations. This is the premise of citric acid accumulation, although the capacity to produce of citric acid is influenced by other culture parameters. Selecting the proper culture conditions is the first step to improve yeast performance.

Most authors report that citric acid production is performed under nitrogen limitation, however some described citric acid production under limitation of phosphorus, sulfur or magnesium. Considering the citric acid production by *Y. lipolytica* IMK 2 growing under nitrogen,

phosphorus, sulfur or magnesium limitation, citric acid accumulation was observed in all media. However, a highest concentration was obtained under nitrogen and sulfur deficient medium (Mckay *et al.*, 1994). Also Kamzolova *et al.* (2011b) reported citric acid production in similar amounts under nitrogen, phosphorus and sulfur limitation, although when the yeast growth was limited by phosphorus or sulfur an increase of isocitric acid was observed.

Carbon source concentration and carbon/nitrogen ratio (C/N) are other factors that can affect the citric acid production. Antonucci *et al.* (2001) study the effect of initial concentration of glucose on citric acid. Working at high carbon concentration was beneficial for specific productivity rate but had a negligible effect on the isocitric acid productivity rate. Also, André *et al.* (2009) reported that increasing carbon source (crude glycerol) from 70 $g \cdot L^{-1}$ to 120 $g \cdot L^{-1}$, maintaining the nitrogen concentration, resulted in an improvement of citric acid concentration from 28 $g \cdot L^{-1}$ to 51 $g \cdot L^{-1}$. Moreover, Levinson *et al.* (2007) tested C/N molar ratio ($mol \cdot mol^{-1}$) between 86 and 1714 and observed that C/N ratios between 343 and 686 led to higher citric acid concentrations. The use of less refined carbon sources can influence cell metabolism and citric acid production. In the specific case of crude glycerol, a few authors compared the production of citric acid using pure and crude glycerol. The citric acid concentration obtained in medium with crude glycerol was slightly lower than in medium with pure substrate. The amount of isocitric acid is also affected, being a little higher in crude glycerol medium (Rywinska *et al.*, 2009; Kamzolova *et al.*, 2011b; Morgunov *et al.*, 2013). Despite the decrease of citric acid amount produced from crude glycerol, it is still satisfactory, so this agro-industrial waste is considered a good carbon source to be used in this process.

Moreover, pH strongly affects yeast growth and metabolism and assumes a crucial role on this citric acid production. Papanikolaou *et al.* (2002a) studied the production of citric acid by *Y. lipolytica* LGAM S (7) using a buffered and non-buffered medium, with an initial pH equal to 6. In the buffered medium (where the pH dropped to 4.5), a 10-fold improvement in citric acid concentration was reached compared to non-buffered medium (where the pH dropped to 2 - 3). The negative effect of low pH can be justified by its influence on citric acid transport across the cell membrane. Anastassiadis and Rehm (2005) studied the effect of pH on the active citric acid transport and demonstrated that this transport system is pH-dependent. Karasu-Yalcin *et al.* (2010) tested different initial pH (between 4.2 and 8.5) and observed that maximum citric acid production was obtained in the range of 5.2 to 7. Studies performed by Tomaszewska *et al.*

(2014) demonstrated that the maximum citric acid concentration was obtained at pH of 5.5 and lower pH values (3 - 4) favored the production of sugar alcohols (erythritol and mannitol).

As citric acid production by *Y. lipolytica* is an aerobic process, oxygen availability is a key parameter in the yeast cultivation and citric acid accumulation (Workman *et al.*, 2013). Studies have shown that higher citric acid productions were obtained when high oxygen concentrations were available in the culture medium (Rywińska *et al.*, 2012). Rywińska *et al.* (2012) also observed an improvement of citric acid production by increasing the agitation rate from 400 rpm to 900 rpm, and the aeration rate from 0.18 vvm to 0.6 vvm. Moreover, dissolved oxygen concentration in the medium directly influences the amount and type of organic compounds produced by the yeast (Okoshi *et al.*, 1987; Finogenova *et al.*, 1991; Rywińska *et al.*, 2012; Kamzolova *et al.*, 2013). Workman *et al.* (2013) demonstrated that in *Y. lipolytica* cultures mannitol and arabitol were produced from glycerol when oxygen limitation occurred. Additionally, it was reported that the increase of dissolved oxygen in glycerol media led to an improvement of citric acid production and to a reduction of isocitric/citric acid ratio (Rywińska *et al.*, 2012; Kamzolova *et al.*, 2013). Kamzolova *et al.* (2003) described that raising dissolved oxygen concentration from 5 % to 60 % of saturation resulted in an increase of citric acid concentration. However, the authors observed that in presence of high iron concentrations it was possible to achieve greater amounts of citric acid with less oxygen concentration (20 %). Other studies, also describe an important effect of salts concentration on yeast growth and citric acid production. Finogenova *et al.* (2002) observed that ion zinc in limited concentrations reduces the cellular growth and citric acid production. Karasu-Yalcin *et al.* (2010) reported that the supplementation of culture medium with zinc had distinct effects on citric acid production by two *Y. lipolytica* strains: decreased with *Y. lipolytica* NBRC 1658 and increased with *Y. lipolytica* NBRC 57.

2.5.2 Operation in bioreactors

The most common reactors used in fermentations and cell cultivations are stirred tank bioreactors. In these bioreactors, air is injected at the bottom of the tank and a Rushton turbine is normally located immediately above the air injector to reduce the bubbles size increasing the oxygen transfer rate (OTR) to liquid phase. It is possible to control aeration and agitation rates to maintain a constant dissolved oxygen concentration in the medium.

Most of studies describing citric acid production by *Y. lipolytica* from crude glycerol use stirred tank bioreactors. These works are summarized in table 2.5 (chapter 2.4). Using this bioreactor type, different operation modes were studied in citric acid production form crude glycerol by *Y. lipolytica* namely, batch, fed-batch, repeated-batch, cell recycle and continuous mode.

Rywińska *et al*. (2010) compared citric acid production of different *Y. lipolytica* strains in batch mode. The strains A-101, Wratislavia 1.31, Wratislavia AWG7 and Wratislavia K1 achieved yields of 0.44 $g \cdot g^{-1}$, 0.41 $g \cdot g^{-1}$, 0.40 $g \cdot g^{-1}$ and 0.25 $g \cdot g^{-1}$, respectively. The strains Wratislavia AWG7 and Wratislavia K1 were used in a different studies using a fed-batch mode and the yields obtained were 0.69 $g \cdot g^{-1}$ and 0.45 $g \cdot g^{-1}$, respectively (Rywińska *et al*., 2009). The fed-batch mode improved the yield of both strains. The strain Wratislavia AWG7 also used by Rywińska and Rymowicz (2011) in continuous mode. The yield in continuous mode was 0.54 $g \cdot g^{-1}$, this value was higher than in batch mode but lower than in fed-batch cultures.

Rymowicz et al. (2010), performed batch, repeated-batch and repeated-batch and cell-recycle to the production of citric acid by *Y. lipolytica* A-101-1.22. In the repeated-batch after a batch for 72 h a volume was withdrawn and replaced with the same volume of fresh medium four times. In the cell recycle mode a continuous operation was carried out using a spiral membrane in the bioreactor, which kept all the cells inside the bioreactor. The yields obtained were 0.66 $g \cdot g^{-1}$, 0.77 $g \cdot g^{-1}$ and 0.64 $g \cdot g^{-1}$ for batch, repeated-batch and cell recycle modes, respectively.

Airlift bioreactors are pneumatically agitated with unique hydrodynamic characteristics and are often employed in bioprocesses where gas-liquid mass transfer is an important parameter (Merchuk *et al*., 1994). Airlift bioreactor was used in two studies of citric acid production by *Y. lipolytica*. This type of bioreactor was applied only with immobilized cells of *Y. lipolytica* and using glucose as carbon source (Kautola *et al*., 1991; Rymowicz *et al*., 1993).

2.5.3 Improvement of strain

Besides the operational and media conditions, the amount of citric acid produced is also highly dependent on the microbial strain. This strain-dependence of citric acid production has been confirmed in some screening-strains studies, comparing citric acid production capability of

strains isolated from natural habitats (Wojtatowicz *et al.*, 1991; Kamzolova *et al.*, 2005; Levinson *et al.*, 2007; Papanikolaou *et al.*, 2009; Kamzolova *et al.*, 2011b). To enhance the citric acid process an improvement of *Y. lipolytica* strains was explored. This improvement has been carried out by mutagenesis/ selection and by genetic engineering transformation.

2.5.3.1 By Mutagenesis

The most employed technique used to improve *Y. lipolytica* strains has been mutation using chemical or physical mutagens. The mutagens usually used are ultraviolet(UV)-irradiation or γ-irradiation, as physical mutagens, different chemical mutagens, or a combination of both mutagens can also be applied.

One of the first studies inducing mutations to improve citric acid production was performed by Hamissa *et al.* (1982). *Candida lipolytica* Y-1095 was exposed to ultraviolet (UV) - irradiation or N-methyl-N'-nitro.N.nitrisoguanidine (NG). The mutants tested to citric acid production display a wide range of results, some strains produce less citric acid and a few achieved higher concentrations compared with the original strain. The four mutants selected in this work show an increase of 75 % to 80 % on citric acid yield. Finogenova *et al.* (2008) work aimed to select mutant strains of *Y. lipolytica* with a high ability to produce citric acid. To obtain the mutants, *Y. lipolytica* YKM Y-2373 was exposed to UV-irradiation, treated with a chemical mutagenic NG, and exposed to both mutagens. Three ace^- mutant strains treated with only one mutagen were selected and presented 23 % more citric acid produced that the parental strain. Additionally, combining both treatments, other three strains were isolated producing more 43.9 % of citric acid than original strain. Moreover, these six strains selected also displayed a lower isocitric acid proportion comparing with the parental strain. Other study was done by Karasu-Yalcin (2012), *Y. lipolytica* 57 was expose to UV irradiation and/or ethyl methane sulfonate (EMS). The isolated strains, incapable to growth in acetate, were selected and their ability to produce citric acid was evaluated. The isolated strain *Y. lipolytica* K-168, resultant from exposition of the original strain to EMS, showed a 57 % increase on citric acid concentration comparing with the initial strain.

2.5.3.2 By Genetic Engineering

The capacity of *Y. lipolytica* to highly express and secrete proteins triggered the development of genetic tools and this yeast started to be used as host to heterologous proteins expression (Fickers, *et al.*, 2005). Nowadays, there are several genetic and molecular tools developed and available for the transformation of *Y. lipolytica* strains.

Few authors already described some important enzymes, from TCA and glyoxylate cycles, involved in citric acid accumulation, like citrate synthase, aconitase hydrate (aconitase), isocitrate lyase, NAD^+-dependent isocitrate hydratase, citrate lyase (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2003). Considering this information, a few set of studies have been performed using genetic transformation in order to understand the influence of key enzymes in the citric acid production of citric acid, and how they affect the isocitric/citric acid ratio (Förster *et al.*, 2007a; 2007b; Holz *et al.*, 2009; Liu *et al.*, 2013; Celińska and Grajek, 2013).

Förster *et al.* (2007b) studied the influence of the overexpression of *ILC1* gene (gene that ecodes isocitrate lyase enzyme) in the isocitric/citric acid ratio. This work demonstrated that these *ILC1* overexpressing strain presented a higher isocitrate lyase activity and a strongly reduced isocitric acid proportion. The proportions of isocitric acid produced were reduced from 10 - 12 % to 3 - 6% using glucose, sucrose or glycerol as carbon source and from 35 – 45 % to 4 - 7 % in hydrophobic substrates (sunflower oils and hexadecane). Moreover, using a defective *ilc1* allele strain a moderated increase on the amount of isocitric acid was reported, proving the impact of isocitrate lyase on isocitric/citric ratio (Förster *et al.*, 2007b).

A similar study was done by Holz *et al.* (2009), that evaluate the influence of *ACO1* gene overexpression (gene that encodes aconitase hydrate) in the isocitric/citric acid ratio. The increase on aconitase activity in *ACO1* overexpressing strains resulted in an increase on isocitric acid proportion. This increase on isocitric acid proportion is more accentuated for sunflower oil, (from 35 - 45 % to 66 - 71 %) in contrast with the moderated increase for other carbon sources tested. For glucose, sucrose and glycerol the rise on isocitric amount was from 10 - 12 % to 13 - 17 % (Holz *et al.*, 2009).

Considering the role of ATP-citrate lyase and isocitrate lyase enzyme in the cell metabolism, Liu *et al.* (2013) increased the copy number of isocitrate lyase gene (*ILC1*) and ATP-citrate lyase genes (*ACL1*) was partially disrupted. The selected strain produced 84.0 $g \cdot L^{-1}$ of

citric acid and only 1.8 g·L^{-1} of isocitric acid, compared to 68.9 g·L^{-1} and 4.1 g·L^{-1} of citric and isocitric acid produced by the parental strain, respectively.

In addition to the modifications made directly in the enzymes involved in the TCA cycle, few studies related to the improvement of carbon source utilization were reported. *Y. lipolytica* is unable to grow in media containing sucrose, and to allow the use of this sugar as a carbon source, strains containing heterologous *scSUC2* gene encoding an invertase were generated by (Förster *et al.*, 2007a). In the same study, the strains were also transformed with isocitrate lyase gene (*ICL1*). This final strain produces large amounts of citric acid (140 g·L^{-1}) and due to the increase of isocitrate lyase activity a lower amount of isocitric acid (< 5 %) was produced.

Recently, Celińska and Grajek (2013) transformed a *Y. lipolytica* strain in order to modify the catabolism of glycerol of this yeast. The constructed strain was transformed with three heterologous genes, encoding a glycerol dehydratase, its reactivator and a wide-spectrum alcohol oxidoreductase controlled by a glycerol-induced promoter. The recombinant strain showed a higher biomass yield and a higher citric acid concentration (59 g·L^{-1}) compared with the host strains (10 g·L^{-1}).

3 OPTIMIZATION OF OPERATING CONDITIONS FOR CITRIC ACID PRODUCTION FROM GLYCEROL BY *YARROWIA LIPOLYTICA* STRAINS

The optimal amount of citric acid produced by *Yarrowia lipolytica* is dependent on yeast strain and growth conditions such as pH, oxygen availability and medium composition. In this work, an experimental design based on Taguchi method was applied to evaluate the effect of the factors pH, carbon/nitrogen (C/N) ratio in the medium, oxygen mass transfer rate (OTR) and salts concentration on citric acid production by two *Y. lipolytica* strains, W29 (ATCC 20460) and CBS 2073. OTR and pH were the factors with more influence on citric acid production for both strains. The increase of OTR from air to culture medium led to 2- and 3-fold improvement of citric acid production by *Y. lipolytica* CBS 2073 and W29, respectively. Besides the individual effect of the factors an important influence of interaction between the factors was observed, mainly between OTR and salts. Different values of factors levels were found as the optimal for each strain, but the theoretically predicted and experimentally obtained concentrations of citric acid were around 10 $g \cdot L^{-1}$ for both strains. The optimal conditions were also validated with crude glycerol from biodiesel industry and similar behavior of the strains was observed using this low cost substrate.

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3.1 INTRODUCTION

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid), an organic acid intermediate of tricarboxylic acids cycle, is largely used in several industries (Kamzolova *et al.*, 2008) and is mainly produced by *Aspergillus niger* from molasses (cane and beet) (Förster *et al.*, 2007a). Although some yeasts are described as citric acid producers, *Yarrowia lipolytica* was already described as citric acid producer from several carbon sources (Arzumanov *et al.*, 2000), (Venter et al., 2004; Kamzolova et al., 2008), glucose (Kamzolova *et al.*, 2008) (Crolla and Kennedy, 2004) and glycerol (Rymowicz et al., 2010), including crude glycerol from biodiesel industry (Papanikolaou and Aggelis, 2003b; Chatzifragkou and Papanikolaou, 2012).

The production and accumulation of citric acid by *Y. lipolytica* occurs in nitrogen-limited conditions and excess of carbon source (Papanikolaou *et al.*, 2002a), but other factors, such as pH (Papanikolaou *et al.*, 2002a) and carbon/nitrogen ratio (Levinson *et al.*, 2007) can also influence the amount of citric acid produced. Since *Y. lipolytica* is a strictly aerobic microorganism, the oxygenation of the culture is also a key factor. Studies have shown that higher citric acid productions were obtained when high oxygen concentrations were available in the culture medium (Rywińska *et al.*, 2012). Besides the operational and media conditions, the amount of citric acid produced is also dependent of the microbial strain (Kamzolova *et al.*, 2003).

In this work, the effect of several factors on citric acid production by two strains of *Y. lipolytica* was studied applying the Taguchi method. This fractional factorial design method allows to identify how each factor affects the citric acid production, which factors have more influence on the target response and to predict the optimal values of factors. Thus, growth conditions such as Carbon/Nitrogen (C/N) ratio, salts concentration, pH and oxygen mass transfer rate (OTR) were optimized for the maximization of citric acid production by two *Y. lipolytica* strains. Although several authors have mentioned the individual effect of each factor on citric acid production, reports about the combined effect of these four factors are scarce.

The experimental validation of the culture conditions at the optimal was done using pure and crude glycerol from biodiesel industry.

3.2 MATERIAL AND METHODS

3.2.1 Yeast strains

Two strains of *Yarrowia lipolytica* (W29 (ATCC 20460) and CBS 2073), that were never tested for citric acid production from glycerol, were used in this study. Strains were maintained on yeast extract peptone dextrose agar medium (YPDA) and kept at 4 °C to a maximum of 2 weeks. The YPDA agar medium composition ($g \cdot L^{-1}$) was: peptone 20, glucose 20, yeast extract 10 and agar 20.

3.2.2 Optimization of growth condition - Experimental design

Citric acid production was optimized using the Taguchi method, a fractional factorial experimental design. This method uses orthogonal arrays for the optimization of different parameters studying a few pairs of parameters combinations instead of all the possible combinations, which reduces time and resources. Orthogonal arrays selection is decided according to the parameters number (P) and the variation of levels (L) of each parameter. The experiments number (N) is calculated by the relation N = (L-1)P+1 (Kumar *et al.*, 2015).

The experimental design was performed using a L9 orthogonal array with Qualiteck-4 software (Nutek, Bloomfield Hills, USA). Four factors (C/N ratio, pH, salts concentration and OTR) were combined and varied in three levels. From Qualiteck-4 software a total of 9 experiments were planned. The experiments were performed in 500 mL flasks filled with 200 mL of production medium. Yeast cells were pre-grown in YPG medium (20 $g \cdot L^{-1}$ of glycerol, 20 $g \cdot L^{-1}$ of peptone, 10 $g \cdot L^{-1}$ of yeast extract), centrifuged and resuspended in the production medium (glycerol as carbon source, yeast extract as nitrogen source, $MgSO_4.7H_2O$ 1.5 $g \cdot L^{-1}$, KH_2PO_4 6 $g \cdot L^{-1}$ and Na_2HPO_4 0.5 $g \cdot L^{-1}$). The experiments were performed for both strains.

pH control was carried out by adding KOH (5 M). The C/N ratio (mass of carbon per mass of nitrogen) was obtained varying the glycerol and yeast extract concentrations: 156 (20 $g \cdot L^{-1}$ glycerol/0.5 $g \cdot L^{-1}$ yeast extract), 391 (50 $g \cdot L^{-1}$ glycerol/0.5 $g \cdot L^{-1}$ yeast extract) and 1956 (50 $g \cdot L^{-1}$ glycerol/0.1 $g \cdot L^{-1}$ yeast extract). Experiments were performed at different OTR values of 48, 192 and 576 $mg \cdot L^{-1} \cdot h^{-1}$ achieved at 140 rpm using flasks without baffles, 140 rpm using flasks with

baffles and at 200 rpm using flasks with baffles, respectively. OTR was estimated in blank assays by the sulfite oxidation method as described by Lopes *et al.* (2013). Salts concentration was 0.15 g·L^{-1} $CaCl_2$, 0.15 g·L^{-1} $FeCl_3.6H_2O$, 0.06 g·L^{-1} $MnSO_4.H_2O$, 0.02 g·L^{-1} $ZnSO_4.7H_2O$ for level 3, half of these concentrations for level 2 and for level 1 no salts solution was added.

The response of citric acid concentration obtained in the experimental design was processed in the Qualiteck-4 software with "bigger is better" quality characteristics to evaluate the optimal culture conditions to maximize the citric acid production. These optimal conditions were assessed for both strains using pure and crude glycerol (provided by Prio Energy - Prio Biocombustíveis, SA) that has the following composition by mass: 90.4 % glycerol, 9 % water, 4.9 % NaCl (less than 0.001 % methanol and less than 0.5 % of organic matter (non-glycerol)).

3.2.3 Analytical methods

Samples were collected for analysis of cell concentration (optical density at 600 nm and converted to dry cell mass per liter), glycerol and citric acid concentration. Glycerol concentration was quantified by high-performance liquid chromatography (HPLC) using a Metacarb 67H (Varian) column (300 mm × 7.7 mm) coupled to refractive index (RI) detector (1530, Jasco). The column was eluted with H_2SO_4 5 mM at 0.5 mL·min^{-1} and the column temperature was 60 °C. Citric acid concentration was measured by HPLC using an YMC ODS-Aq (250 x 4.6 mm) reverse phase column coupled to a diode array (DAD) detector at 214 nm. The mobile phase was KH_2PO_4 20 mM, pH 2.8 at room temperature and a rate flow of 0.7 mL·min^{-1}.

3.3 RESULTS AND DISCUSSION

Two strains of *Y. lipolytica* were tested: *Y. lipolytica* W29 (ATCC 20460) and *Y. lipolytica* CBS 2073. *Y. lipolytica* W29 has been successfully used for lipase (Lopes *et al.*, 2008), γ-decalactone (Braga and Belo, 2014) and also for citric acid production from glucose (Sarris *et al.*, 2011). Furthermore, due to the availability of its complete genome sequence, *Y. lipolytica* W29 has been used as a model by several research groups for genetic modifications (Nicaud *et al.*, 2002). There are reports that *Y. lipolytica* CBS 2073 can grow efficiently and produce lipase in an agro-industrial waste (olive mill wastewater) (Gonçalves *et al.*, 2009). This work also intends

to study the use of a crude waste of biodiesel industry (crude glycerol) for citric acid production, which justifies the selection of these strains.

The profile through time for citric acid and glycerol concentration is shown on Figure. 3.1 for run 2 as an example, since identical behavior was observed for all runs. A continuous citric acid increase and glycerol decrease in the medium with time was observed till the end of the assays (168 h). The response of interest was the final citric acid concentration that is shown for all the runs in Table 3.1.

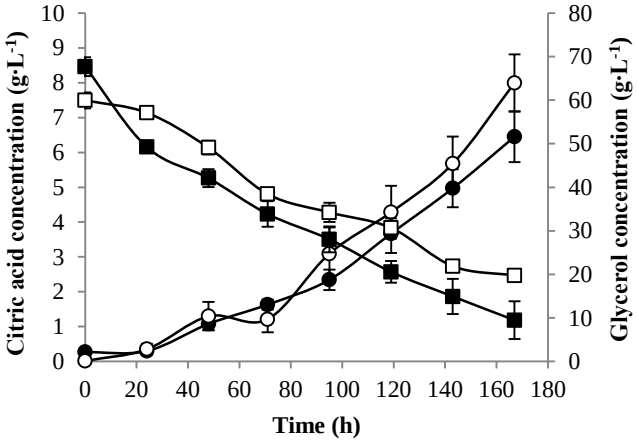

Figure 3.1 Time course of citric acid production (●,○) and glycerol consumption (■,□) for *Y. lipolytica* W29 (closed symbols) and *Y. lipolytica* CBS 2073 (open symbols) from Run 2. pH = 5, C/N = 391, OTR = 192 $mg \cdot h^{-1} \cdot L^{-1}$ and ½ salts concentration. The error bars represent the standard deviation for two independent replicates.

From the responses obtained for all the experiments it is clear that citric acid production is strongly dependent on the combination of the various factors studied. The citric acid concentration varied from 0.1 $g \cdot L^{-1}$ to 8.1 $g \cdot L^{-1}$.

Table 3.1 Factors and levels used in the experimental design for each assay performed and citric acid concentration obtained in the experiments designed using Taguchi L9 orthogonal array in batch cultures of *Y. lipolytica* W29 and CBS 2073. Data are the average and standard deviation of two independent replicates.

					Citric acid concentration ($g \cdot L^{-1}$)	
					Y. lipolytica	
Run	**pH**	**C/N ratio**	**OTR**	**Salts**	**W29**	**CBS 2073**
1	1	1	1	1	3.7 ± 0.2	3.5 ± 0.1
2	1	2	2	2	6.2 ± 0.8	7.0 ± 0.2
3	1	3	3	3	6.2 ± 0.1	4.7 ± 0.2
4	2	1	2	3	2.6 ± 0.2	8.1 ± 0.7
5	2	2	3	1	4.4 ± 0.3	4.5 ± 0.4
6	2	3	1	2	2.0 ± 0.2	2.8 ± 0.1
7	3	1	3	2	4.0 ± 0.4	2.0 ± 0.0
8	3	2	1	3	0.1 ± 0.1	2.2 ± 0.0
9	3	3	2	1	0.7 ± 0.1	1.7 ± 0.1
Level						
1	5	156	48	0		
2	6	391	192	½		
3	7	1956	576	1		

The individual effect of each factor on citric acid production is shown in Figure 3.2. The increase of pH value from 5 to 7 led to a decrease on citric acid concentration, particularly for *Y. lipolytica* W29. In the experiments carried out at pH 5, citric acid concentration was 3-fold higher than that obtained at pH 7. However for CBS strain no significant differences were obtain between pH 5 and 6. The effect of C/N ratio was similar for both strains; a small increase was attained with a ratio equal to 391. In the experiments with *Y. lipolytica* W29, citric acid production increased proportionally with OTR and the raise of OTR from level 1 to 3 led to a 3-fold improvement in citric acid concentration. However, for *Y. lipolytica* CBS 2073, the best condition was in the intermediate OTR level where a 2-fold increase of citric acid production was obtained compared with the result at the lowest OTR value. The raise of salts concentration in the culture medium had a slight positive effect on citric acid production for both strains but for *Y. lipolytica* CBS 2073 the maximum citric acid was obtained at highest concentration of salts, while for *Y. lipolytica* W29 half of salts concentration was enough to reach the maximum citric acid production.

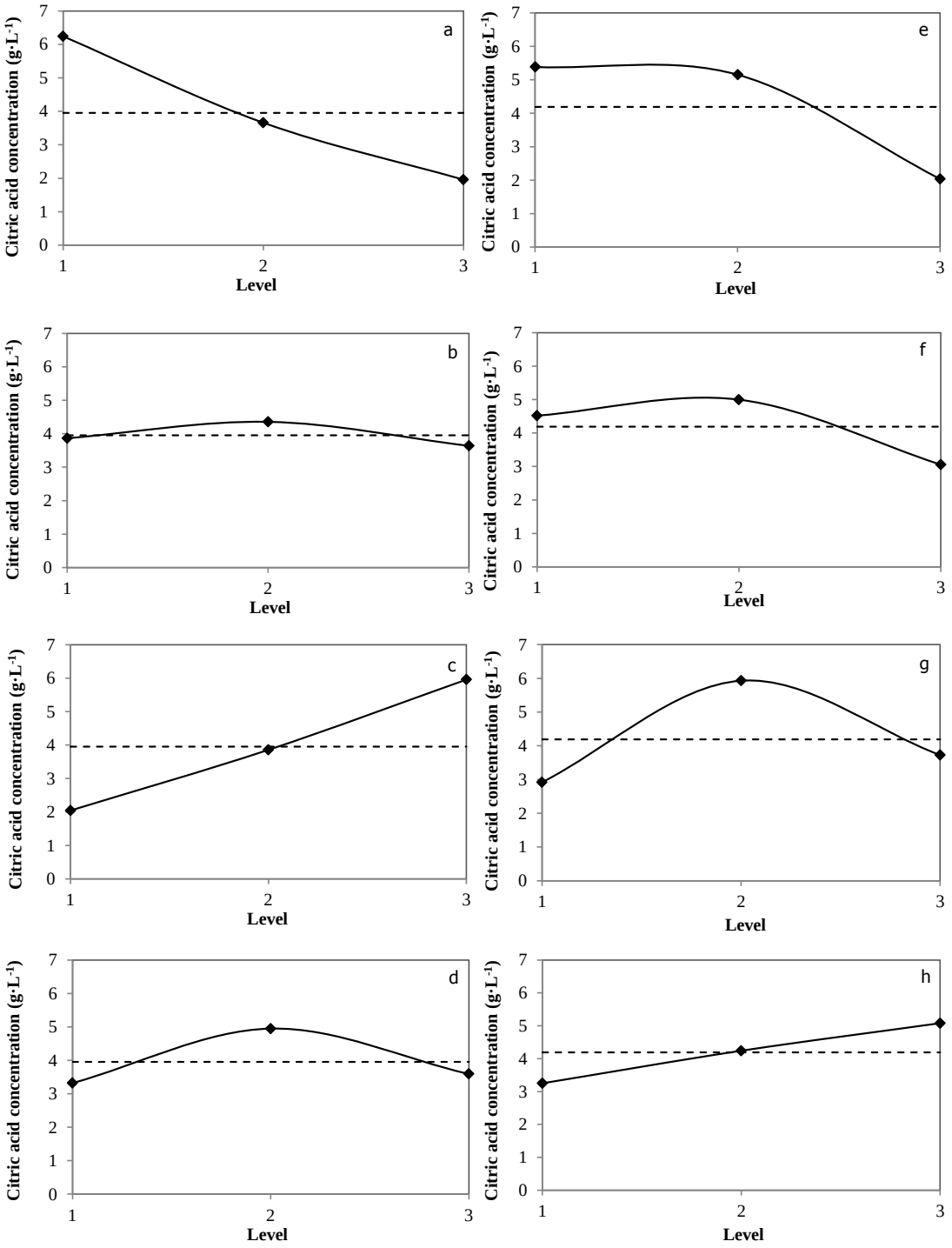

Figure 3.2 Individual factors (pH – a, e; C/N ratio – b, f; OTR- c, g; salts – d, h) effect at different levels for *Y. lipolytica* W29 (left column) and *Y. lipolytica* CBS 2073 (right column). "Levels" description is shown in Table 1.

The individual contribution of each factor is very important to define the parameters that must be strictly controlled during the production process. The analysis of variance (ANOVA) for

the selected response (Table 3.2) may contribute to understand which factors have more influence on citric acid production by *Y. lipolytica* strains. The last column (P, %) of Table 3.2 indicates the contribution of each factor, being that the higher percentage represents the factor with more influence in the process. According with these results, it was possible to select the factors pH and OTR as the most significant for citric acid production by both yeast strains. Although pH and OTR were by far the most influent parameters on citric acid production for both strains, the best level of each one was slightly different. This result proves that optimal conditions are dependent on yeast strain.

Papanikolaou *et al.*, (2002a) studied the production of citric acid by *Y. lipolytica* LGAM S (7)1 using a buffered and non-buffered medium, with an initial pH of 6. In the buffered medium (where the pH dropped to 4.5), a 10-fold improvement in citric acid concentration was reached compared to non-buffered medium (where the pH dropped to 2 - 3). Karasu-Yalcin *et al.* (2010) tested different initial pH (between 4.2 and 8.5) and observed that maximum citric acid production was obtained in the range of 5.2 to 7. Studies performed by Tomaszewska *et al.* (2014) demonstrated that the maximum citric acid concentration was obtained at pH 5.5 and lower pH values (3 - 4) favored the production of sugar alcohols. The negative effect of low pH can also be justified by it influence on citric acid transport across the cell membrane. Anastassiadis and Rehm (2005) studied the effect of pH on the active citric acid transport and demonstrated that this transport system is pH-dependent.

Table 3.2 Analysis of variance (ANOVA) for the Taguchi L9 orthogonal array.

Strains	Factor	Sum of squares	Variance	F-Ratio	P (%)
Y. lipolytica W29	pH	55.86	27.93	106.48	48.13
	C/N ratio	1.62	0.81	3.08	0.95
	OTR	46.02	23.01	87.73	39.57
	Salts	9,12	4.56	17.38	7.47
	Error	2.36	0.26	-	3.88
Y. lipolytica CBS 2073	pH	51.00	25.50	37.31	45.82
	C/N ratio	9.52	4.76	6.96	7.53
	OTR	34.70	17.35	25.39	30.78
	Salts	6.94	3.47	5.08	5.15
	Error	6.15	0.68	-	10.73

The oxygen has also been reported as an important factor for citric acid production. Rywińska *et al*. (2012) observed an improvement of citric acid production increasing the agitation rate from 400 rpm to 900 rpm, and the aeration rate from 0.18 vvm to 0.6 vvm, but no information on OTR was given.

In this work, the salts concentration in the production medium was a factor with little influence on citric acid production. However, some studies have shown that salts concentration can have an important role on yeast growth and citric acid production. Finogenova *et al*. (2002) observed that ion zinc in limiting concentrations reduces the cellular growth and citric acid production. It was demonstrated in this work, that the effect of salts concentration is also dependent of *Y. lipolytica* strain. Similarly, Karasu-Yalcin *et al*. (2010) reported that the supplementation of culture medium with zinc had distinct effects on citric acid production by two *Y. lipolytica* strains: decreased with *Y. lipolytica* NBRC 1658 and increased with *Y. lipolytica* NBRC 57.

The production of citric acid occurs in nitrogen-limited conditions and excess of carbon source. In this study, C/N ratio is one of the factors with less influence on citric acid production and a ratio equal to 391 led to a higher concentration of citric acid. There are some studies showing that greater citric acid concentrations were also reached with higher C/N ratios. Levinson *et al.* (2007) observed that C/N ratios between 343 and 686 led to higher citric acid concentrations. André *et al*. (2009) also reported that the increase of carbon source (glycerol), maintaining the nitrogen concentration, led to an improvement of citric acid production.

Besides the effect of each factor individually, the interaction between factors can give a better insight into the overall process. Estimated interaction severity index (SI) allows understanding the influence of two factors interaction (Table 3.3). It is worth to notice that the highest severity index is not associated with the most important factors (individual effect) and the severity index values for each factor combination depends on the yeast strain studied. For *Y. lipolytica* W29 the interaction of C/N ratio (factor with little individual effect) *vs* OTR (factor with high individual effect) has the higher severity index (53.45 %), closely followed by salts concentration *vs* OTR (52.23 %). The interaction between the factors that had more influence individually (pH x OTR) presents lower severity index (24.71 %). For *Y. lipolytica* CBS 2073 the interactions with higher severity index were C/N ration *vs* pH (62.67 %) and salts concentration *vs* OTR (54.48 %). It was, observed in other studies that the oxygen requirements for citric acid

production by *Y. lipolytica* are lower in the presence of higher concentrations of iron (Finogenova *et al*., 2002; Kamzolova *et al*., 2003), which are in accordance with the results reported here and demonstrates the importance of salts concentration *vs* OTR interaction. From the analysis of interactions it was observed that the most important ones were between a factor with lower individual effect and a more influent factor. These results suggest that the influence of a factor depends on the conditions of the other factor in the optimization of citric acid production.

Table 3.3 Estimated interactions of studied factors based on severity index (SI %).

Interacting factor pairs	*Y. lipolytica*	
	W29	CBS 2073
C/N ratio *vs* OTR	53.15	9.86
OTR *vs* Salts	52.23	54.48
pH *vs* Salts	46.37	48.67
pH *vs* OTR	24.71	6.51
pH *vs* C/N ratio	10.8	62.67
C/N ratio *vs* Salts	5.7	38.8

Taking into account the experimental data obtained, the Taguchi method established the optimal level of each factor for maximization of citric acid production and predicted a theoretical value in optimal conditions (Table 3.4). pH and C/N ratio are the same for both strains, despite the OTR and salt concentration needed to maximized citric acid concentration for each strain being different. *Y. lipolytica* W29 needs a higher OTR value but lower salt concentration when compared with *Y. lipolytica* CBS 2073. The importance of OTR and salts interaction was reported by Finogenova *et al*. (2002) and Kamzolova *et al*. (2003). The authors observed that, in presence of high iron concentrations it was possible to achieve great amounts of citric acid with less quantity of oxygen.

In order to confirm the theoretical values and validate the experimental design, assays were carried out in optimal conditions predicted by the method for both strains (Table 3.4). For both strains, the experimental and predicted results were similar, validating the method and allowed establishing the optimal conditions for citric acid production by these *Y. lipolytica* strains.

Table 3.4 Optimal culture conditions, predicted and experimental citric acid concentrations obtained for batch cultures of *Y. lipolytica*. Data are the average and standard deviation of two independent replicates.

Strains	Factor	Level	Values	Predicted results ($g \cdot L^{-1}$)	Experimental results ($g \cdot L^{-1}$)
Y. lipolytica W29	pH	1	5	9.6	9.5 ± 0.6 (pure) 10.3 ± 0.1 (crude)
	C/N ratio	2	391		
	OTR	3	576		
	Salts	2	½		
Y. lipolytica CBS 2073	pH	1	5	8.8	10.5 ± 0.3 (pure) 9.4 ± 0.8 (crude)
	C/N ratio	2	391		
	OTR	2	192		
	Salts	3	1		

Current grand average performance: *Y. lipolytica* W29 - 3.9 $g \cdot L^{-1}$; *Y. lipolytica* CBS 2073 - 4.2 $g \cdot L^{-1}$

Besides the assay with pure glycerol, an experiment with crude glycerol at optimal conditions for both strains was performed. As the main by-product of biodiesel production, crude glycerol can now be found in abundance and at prices lower than pure glycerol, which makes possible to use crude glycerol as carbon source for bioprocesses with *Y. lipolytica*. There were no statistical differences ($p > 0.05$) on citric acid production with pure and crude glycerol (Table 3.4), which indicates that the impurities and additional nutrients present in crude glycerol do not affect the citric acid production by both *Y. lipolytica* strains used in this work. The current low cost of crude glycerol together with the present results shows the possibility of using crude glycerol as carbon source for citric acid production by *Y. lipolytica*. As with pure glycerol, the citric acid concentrations obtained in the optimal culture conditions with crude glycerol are not statistically different between the strains ($p > 0.05$).

3.4 CONCLUSIONS

Taguchi experimental design for process optimization allowed to conclude that pH and OTR are the factors with more influence on citric acid production in batch cultures of two *Y. lipolytica* strains, W29 and CBS 2073, using glycerol as substrate. Moreover, a significant

interaction with OTR and salts concentration was found for both strains. These are important parameters for process scale-up.

The optimal conditions to maximize the citric acid concentration were: pH 5 and C/N ratio of 391 ($g \cdot g^{-1}$) for both stains, OTR of 576 $mg \cdot L^{-1} \cdot h^{-1}$ and half of the salts concentration for *Y. lipolytica* W29, and OTR of 192 $mg \cdot L^{-1} \cdot h^{-1}$ and full concentration of salts for *Y. lipolytica* CBS 2073. The production of citric acid was similar for both strains, but in a larger-scale production, W29 strain allows the use of smaller amounts of salts, provided that a good oxygen transfer into the medium is ensured. A good oxygenation of the medium is easily obtained with several bioreactors used in industry, such as stirred tank bioreactors. For this reason, only *Y. lipolytica* W29 strain was used in the following work described in next chapters.

The citric acid concentrations obtained for both stains using crude glycerol from biodiesel industry were similar to those obtained with pure glycerol, validating the possibility of using this byproduct as a low cost carbon source for citric acid production by the *Y. lipolytica* strains used in this work.

4 CITRIC ACID PRODUCTION BY *YARROWIA LIPOLYTICA* FROM CRUDE GLYCEROL IN A STIRRED TANK BIOREACTOR: OXYGEN MASS TRANSFER IMPACT

Production of citric acid from crude glycerol, in batch cultures of *Yarrowia lipolytica* W29 was performed in a lab-scale stirred tank bioreactor in order to assess the effect of oxygen mass transfer rate in this bioprocess. An empirical correlation was proposed to describe oxygen volumetric mass transfer coefficient (k_La) as a function of operating conditions (stirring and aeration rates) and cellular density. k_La increased according with a power function with specific power input and superficial gas velocity, and slightly decreased with cellular density. The increase of k_La from 7 h^{-1} to 55 h^{-1} led to 7.8-fold increase of citric acid final concentration. Experiments were also performed at controlled dissolved oxygen (DO) and citric acid concentration increased with DO up to 60 % of saturation. Thus, due to the simpler operation at constant k_La than at controlled DO, it can be concluded that k_La is an adequate parameter for the optimization of citric acid production from crude glycerol by *Y. lipolytica* W29 and to be considered in bioprocess scale-up. Our empirical correlation, considering the operating conditions and cellular density, will be a valid tool for this purpose.

The information presented in this Chapter was submitted to *Biochemical Engineering Journal:*

Ferreira, P., Lopes, M., Mota, M., Belo, I., Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol. (October 2015).

4.1 INTRODUCTION

Yarrowia lipolytica is strictly aerobic and some studies have already showed the effect of oxygen mass transfer rate on yeast metabolism and products formation. The raise of oxygen transfer from gas phase to the culture medium resulted in an increase of cellular growth (Lopes *et al.*, 2009), lipase production (Lopes *et al.*, 2008) and γ-decalactone secretion (Braga *et al.*, 2015). As citric acid production by *Y. lipolytica* from glycerol is an aerobic process, oxygen availability is a key parameter in the yeast cultivation, substrate uptake rate and citric acid accumulation (Workman *et al.*, 2013). Moreover, dissolved oxygen concentration in the medium could directly influence the amount and type of organic compounds produced by the yeast (Okoshi *et al.*, 1987; Finogenova *et al.*, 1991; Rywińska *et al.*, 2012; Kamzolova *et al.*, 2013). Workman *et al.* (2013) demonstrated that in *Y. lipolytica* cultures mannitol and arabitol were produced from glycerol when oxygen limitation occurred. Additionally, it was reported that the increase of dissolved oxygen in glycerol media led to an improvement of citric acid production and to a reduction of isocitric/citric acid ratio (Rywińska *et al.*, 2012; Kamzolova *et al.*, 2013), but no reference of oxygen mass transfer rate was reported.

Oxygen is a key substrate in any aerobic bioprocess since it is an important nutrient for microbial growth, maintenance and metabolites production. Thus, a continuous supply of oxygen to the culture broth is needed due to its low solubility in aqueous medium (Garcia-Ochoa and Gomez, 2009). It is very important to know and, if possible, to predict the oxygen mass transfer rate (OTR) and volumetric oxygen mass transfer coefficient ($k_L a$) for different operating conditions to ensure sufficient oxygen transfer from the gas phase to the culture medium. OTR can be affected by several factors, such as geometrical characteristics of the bioreactor, operating conditions, physical properties of gas and liquid phases and by the presence of cells (Garcia-Ochoa and Gomez, 2009; Suresh *et al.*, 2009). OTR and $k_L a$ can be related by equation 4.1:

$$OTR = k_L a(C^* - C) \qquad \text{Eq. 4.1}$$

where $k_L a$ is the mathematical product of mass transfer coefficient (k_L) and interfacial area (a), C* is the solubility of oxygen and C is the dissolved oxygen concentration in the liquid phase.

Numerous empirical correlations have been proposed to calculate $k_L a$, depending on the bioreactor configuration (Garcia-Ochoa and Gomez, 2009). For a stirred tank bioreactor (STR) the most common function is given by equation 4.2 (Cooper *et al.*, 1944):

$$k_L a = \alpha \left(\frac{P_g}{V}\right)^{\beta} v_s^{\gamma} \qquad \text{Eq. 4.2}$$

where P_g is the power input to the aerated system, V is the working volume, v_s represents the superficial gas velocity and α, β and γ are dimensionless constants.

In order to estimate the power input to the aerated system (P_g), the Reynolds number (Re) is determined by equation 4.3 and the power number (N_p) by equation 4.4:

$$Re = \frac{D_i^2 N \rho}{v} \qquad \text{Eq. 4.3}$$

$$N_p = \frac{P_g}{\rho N^3 D_i^5} \qquad \text{Eq. 4.4}$$

where D_i represents the impeller diameter, N the stirring rate, ρ the liquid density and v the liquid viscosity.

If the Reynolds number is between 19070 and 38141 the flow regime inside the system is considered turbulent and N_p is not a function of Re (Cheremisinoff and Gupta, 1983). Thus, P_g without aeration (P_g') can be calculated by equation:

$$P_g' = K_T D_i^5 N^3 \rho \qquad \text{Eq. 4.5}$$

where K_T represents a constant dependent on the impeller.

Finally, P_g in the aeration system is determined by equation 4.6:

$$P_g = c \left(\frac{P_g' N D_i^3}{F_g^{0.56}}\right)^{0.45} \qquad \text{Eq. 4.6}$$

where c represents a constant that depends on the impeller used and F_g is the volumetric gas flow rate.

Although several works regarding the production of citric acid from crude glycerol have been published, data on oxygen volumetric mass transfer rate and dissolved oxygen required to support the production in this medium are still limited. Moreover, there is a lack of correlations to predict $k_L a$ in production medium and the effect of active cells on oxygen mass transfer. The

correct prediction of k_La is a crucial step to achieve an optimal operation design and scale-up of bioreactors. Thus, experimental values of k_La were obtained in a lab-scale STR, by varying the stirring and the aeration rates, and its effect on citric acid production was evaluated. Data fitting to an empirical correlation for the prediction of k_La as a function of superficial gas velocity and power input of the aerated bioreactor, based on equation 4.2, was attempted with a correction to predict the effect of cells on k_La.

Finally, the behavior of yeast growth, substrate consumption and citric acid production under constant dissolved oxygen concentration in the medium were analyzed in order to find which intrinsic parameter of oxygenation is more important for citric acid production, k_La or controlled dissolved oxygen (DO). Several batch experiments were performed at 20 %, 40 % and 60 % of dissolved oxygen saturation.

4.2 MATERIALS AND METHODS

4.2.1 Strain and Medium

Y. lipolytica W29 (ATCC 20460) was maintained in YPDA medium (described in chapter 3.2.1) at 4 °C for a maximum of two weeks.

4.2.2 Bioreactor assay

Yeast cells were pre-grown for 18 h in 500 mL Erlenmeyer flask filled with 200 mL of pure glycerol 20 $g{\cdot}L^{-1}$, peptone 20 $g{\cdot}L^{-1}$ and yeast extract 10 $g{\cdot}L^{-1}$ medium, at 27 °C and 200 rpm.

Cells were centrifuged and resuspended in the production medium composed by ($g{\cdot}L^{-1}$): crude glycerol 50; yeast extract 0.5; $MgSO_4{\cdot}H_20$ 1.5; KH_2PO_4 6; Na_2HPO_4 0.5; $CaCl_2$ 0.75; $FeCl_3{\cdot}6H_2O$ 0.75; $ZnSO_4{\cdot}7H_2O$ 0.1; $MnSO_4{\cdot}H_20$ 0.3. Crude glycerol was provided by Prio Energy - Prio Biocombustíveis, SA and has the following composition (w/w): 90.4 % glycerol, 9 % water, 4.9 % NaCl, less than 0.001 % methanol and 0.5 % of organic matter (non-glycerol).

Batch assays were carried out in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 31 cm height and 17 cm diameter, and with Rushton impeller, 6-blade, 6 cm

outside diameter (Figure 4.1). The medium pH was kept at 5 by addition of potassium hydroxide (2 M) or orthophosphoric acid 21 % (v/v), through Peripex peristaltic pumps (Bioengineering, Switzerland). Dissolved oxygen concentration was measured with a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA) using the BioScadaLab software.

The bioreactor, filled with 1.7 L of production medium, was inoculated at an initial cell density of 0.5 $g \cdot L^{-1}$ *Y. lipolytica* cells and the assays were performed at 27 °C.

In order to evaluate the effect of $k_L a$ on citric acid production, several experiments were carried out varying the aeration rate from 1 vvm to 3 vvm and changing the stirring rate from 200 rpm to 600 rpm.

Additionally, several assays with constant dissolved oxygen (20 %, 40 % and 60 %) were performed. The DO concentration in the culture medium was controlled by manipulating the stirring and aeration rates, through a cascade control mode. In the cascade mode, the stirring and aeration rate automatically varied between the values studied in $k_L a$ modeling (200 rpm – 600 rpm of stirring rate and 1 vvm – 3 vvm of aeration rate).

Each experiment was replicated twice to ensure the repeatability and the reproducibility of the results.

Figure 4.1 Stirred tank bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with production medium.

4.2.3 k_La calculation

4.2.3.1 Static gassing-out technique

For experimental k_La determination in blank assays (without cells), the static gassing-out technique was used. This method allows evaluating the effect of operational parameters, such as stirring and aeration rates, in the oxygen transfer efficiency (Wise, 1951). After a preliminary gassing-out with compressed nitrogen to remove the oxygen in the medium, the aeration was switched on at specific conditions of aeration and stirring rates until saturation.

The technique is based in the oxygen mass balance equation (Eq. 4.7) which, in the absence of cells and in batch mode, is simplified to the equality between the time variation of the dissolved oxygen concentration $\left(\frac{dC}{dt}\right)$ and the oxygen transfer rate from the gas to the liquid.

$$\frac{dC}{dt} = k_L a(C^* - C) \qquad \text{Eq. 4.7}$$

Integrating this equation, the value of k_La was obtained, which is equal to the symmetrical slope of the plot of $\ln(C^* - C)$ *vs* time (Stanbury and Whitaker, 1984).

The probe response time (τ) was estimated according to Tribe *et al*, 1995, and a value of 7 s was obtained. k_La values were corrected according to equation 4.8:

$$\frac{1}{k_L a'} = \frac{1}{k_L a} + \tau \qquad \text{Eq. 4.8}$$

where $k_L a'$ is the oxygen volumetric mass transfer coefficient determined experimentally.

4.2.3.2 Dynamic gassing-out technique

During citric acid production by *Y. lipolytica* cells, k_La was determined using the dynamic gassing-out technique. The method is based on following the dissolved oxygen concentration in cultivation medium during a short interruption of the aeration (Bandyopadhay *et al*., 1967). In the presence of active cells and in the absence of aeration, the respiratory activity of yeast cells leads to the removal of oxygen of the liquid medium.

The procedure involves two steps: one to stop aeration and another to restart aeration in the operating conditions. Thus, in the first step, monitoring the decrease of dissolved oxygen concentration will allow to determine the specific oxygen uptake rate (OUR) (Eq. 4.9):

$$\frac{dC}{dt} = -OUR \qquad \text{Eq. 4.9}$$

Aeration is restarted before reaching the critical dissolved oxygen concentration value (Tribe *et al.*, 1995). After the resumption of aeration, the oxygen mass balance in the liquid phase is expressed by equation 4.10:

$$\frac{dC}{dt} = k_L a(C^* - C) - OUR \qquad \text{Eq. 4.10}$$

4.2.3.3 $k_L a$ modeling

To take into account the effect of cellular concentration, X, on $k_L a$, a correction of equation 4.2 was made (Eq. 4.11).

$$k_L a = \alpha \left(\frac{P_g}{V}\right)^{\beta} v_s^{\gamma} (1 + X)^{\delta} \qquad \text{Eq. 4.11}$$

The power input to the aerated system (P_g) and the superficial gas velocity (v_s) were calculated using the equations presented in the introduction, converting the aeration rate to real volumetric gas flow rate (F_g). According to Michel and Miller (1962), the parameters of these empirical equations depend of system geometry and are only valid for superficial gas velocity between 0.042 $m \cdot s^{-1}$ and 0.180 $m \cdot s^{-1}$ and stirring rate between 180 rpm and 960 rpm, that cover the conditions used in this work.

For $k_L a$ modeling, the data fitting to equation 4.11 was performed by least-squares non-linear regression using the Solver tool of Microsoft Excel 2010 software.

4.2.4 Analytical methods

Samples were periodically collected to measure biomass concentration, glycerol consumption, citric acid and isocitric acids production. The samples analyses were performed as described in the chapter 3.2.3. Isocitric acid was quantified by HPLC as citric acid.

4.3 RESULTS AND DISCUSSION

4.3.1 k_La modelling in STR bioreactor

To evaluate the effect of power input in the aerated system, the superficial gas velocity and cell density on k_La values, several experiments were carried out in a 3.7-L stirred tank bioreactor, by changing simultaneously stirring and aeration rates. The experimental results of k_La obtained in the different experimental conditions are presented in Table 4.1. As expected for a STR bioreactor, the increment of stirring and aeration rates led to an enhancement of k_La value. 18-Fold improvement in k_La values was obtained by increasing the aeration rate from 1 vvm to 3 vvm and the stirring rate from 200 rpm to 600 rpm. The k_La experimental value for the assay at 200 rpm of stirring rate and 1 vvm of aeration rate with cells was not possible to calculate, since a total depletion of oxygen through time of production process was recorded.

Table 4.1 Experimental k_La values under different experimental conditions. Data are presented as the average and standard deviation of two independent experiments.

Experimental conditions		k_La (h^{-1})	
Aeration rate (vvm)	Stirring rate (rpm)	Without cells	With cells
1	200	7 ± 1	–
1.5	300	20 ± 1	18 ± 2
2	400	55 ± 3	48 ± 3
2.5	500	84 ± 3	81 ± 1
3	600	128 ± 15	89 ± 3

Several parameters, from fluid properties to bioreactor geometry, can influence the k_La estimation in the system. The determination of k_La is a crucial step in bioreactor design, allowing to quantify the effect of the operating variables on the provision of oxygen and to establish the aeration capacity of the bioreactor system. Using the experimental data obtained in the assays with increased stirring and aeration rates (Table 4.1), in citric acid medium with and without yeast cells, the values of α, β, γ and δ coefficients from equation 4.11 were estimated as indicated in equation 4.12:

$$k_La = 86\left(\frac{P_g}{V}\right)^{0.51} v_s^{0.46}(1+X)^{-0.12} \qquad \text{Eq. 4.12}$$

For the empirical equation, $\frac{P_g}{V}$ values ranged from 7.9 W·m^{-3} to 191.0 W·m^{-3} and v_s from 2.3x10^{-3} m·s^{-1} to 6.9x10^{-3} m·s^{-1}. For aqueous systems, a wide range for the coefficient values were proposed in the literature, in which coefficient of $\frac{P_g}{V}$ varied between 0.3 and 0.8 and coefficient of v_s varied from 0.4 to 1 (Kawase and Moo-Young, 1988; Garcia-Ochoa and Gomez, 2009). These variations resulted from differences in physicochemical properties (ionic strength, viscosity and surface tension) of the culture broths used by the authors. The results obtained in the present work, in a citric acid production medium, show that the k_La dependence is slightly higher on the specific power input than on the superficial gas velocity, once the coefficient of v_s is lower than the coefficient of $\left(\frac{P_g}{V}\right)$. In previous work (Braga *et al.*, 2015), in the same bioreactor but with a biphasic culture medium (oil-in-water emulsion), a same influence of the power input (β of 0.5) was obtained, but a lower influence of superficial gas velocity (α of 0.2) was found.

The presence of cells in the system resulted in a slight negative effect on k_La. Also other authors reported a decrease in the k_La values with the increase in cell concentration (Amaral *et al.*, 2008; Shin *et al.*, 1996). This effect may be explained by the effect of cells as solid particles that may block the transfer of oxygen from air bubbles to the liquid phase (Garcia-Ochoa and Gomez, 2009).

In Figure 4.2, predicted *versus* experimental k_La results are represented with a line slope close to 1, which indicates a good approximation between real k_La values and the values calculated by the correlation.

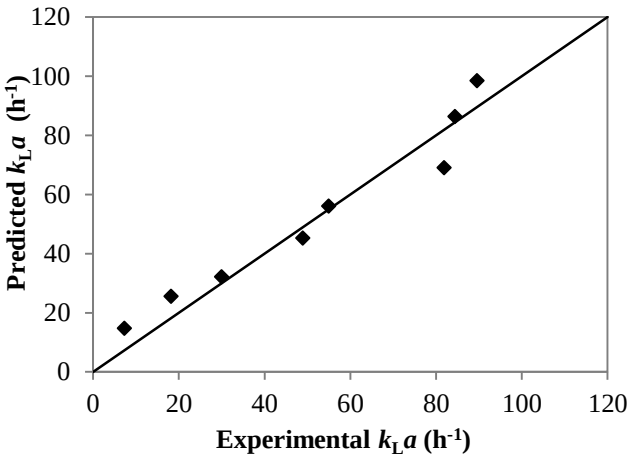

Figure 4.2 Correlation between the experimental and predicted k_La values using equation 4.12.

4.3.2 Effect of k_La on citric acid production

In order to evaluate the effect of k_La on citric acid production, several experiments were performed varying simultaneously the stirring and aeration rates (table 4.1). The raise of k_La from 7 h^{-1} to 125 h^{-1}, due to an increase of aeration and stirring rates, had a clearly positive impact on citric acid production (Figure 4.3c). At lower k_La value (7 h^{-1}) the cells presented longer lag phase than in the higher values, and no significant differences were observed for cellular growth between them (Figure 4.3a). Additionally, the crude glycerol consumption profile was similar for all the experiments, with exception for the assay at lower k_La value (Figure 4.3b).

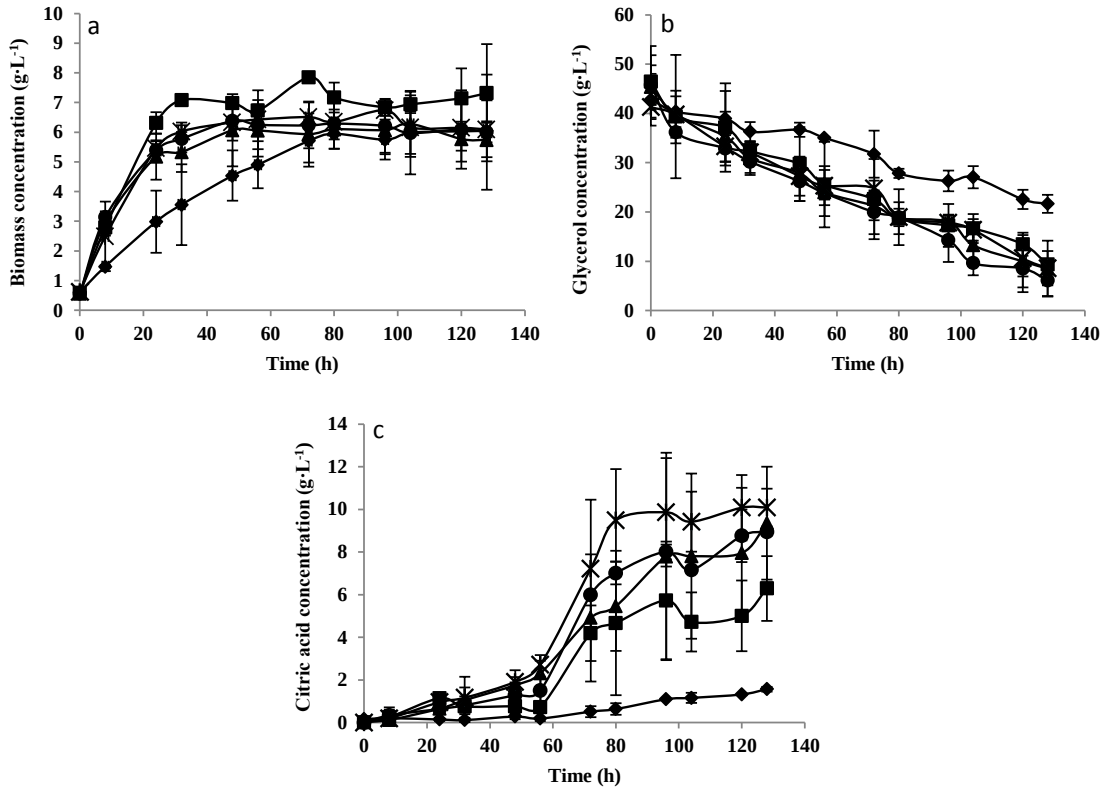

Figure 4.3 Biomass production (a), crude glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 at different k_La values (h^{-1}): 7 (♦), 30 (■), 55 (▲), 84(●) and 125 (×).The error bars represent the standard deviation of two independent replicates.

As expected, according with k_La conditions, different dissolved oxygen profiles were observed in batch cultures of *Y. lipolytica* (Figure 4.4). During the first hours of yeast cultivation (corresponding to the exponential growth phase) a decrease on oxygen concentration in the medium was observed, particularly in the experiments with lower values of k_La (7 h^{-1} and 30 h^{-1}). In fact, for a k_La value of 7 h^{-1}, a completely depletion of oxygen from the medium was observed through all the process, which can justify the lower biomass and citric acid concentrations obtained in this condition. In the phase of citric acid production (after the nitrogen source had been completely consumed), the oxygen demand is lower and a raise of oxygen concentration in the medium has been reported (Rane and Sims, 1994; Wentworth and Cooper, 1996; Rywińska *et al.*, 2012). In the experiments with a k_La value equal to 30 h^{-1}, the dissolved oxygen concentration dropped to zero in the first hours but stabilized around 20 % during the citric acid production. For the other k_La conditions, the oxygen concentration in the medium never fell to

zero and stabilized around 55 %, 70 % and 85 % for k_La values of 55 h^{-1}, 84 h^{-1} and 125 h^{-1}, respectively.

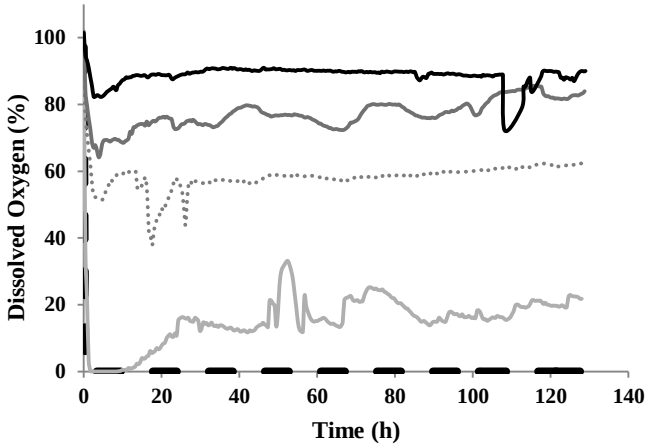

Figure 4.4 Dissolved oxygen concentration profiles during citric acid production in batch cultures of *Y. lipolytica* W29 at different k_La values (h^{-1}): 7 (dashed line); 30 (light grey line); 55 (dotted line); 84 (dark grey line); 125 (black line).

The raise of k_La from 7 h^{-1} to 55 h^{-1} led to an increase of citric acid concentration and maximum productivity (Figure 4.5). Approximately 8-fold improvement in citric acid concentration and maximum productivity was observed. The lower concentration was attained in the experiments where a completely oxygen depletion from the medium was observed and the highest citric acid concentration was reached in the experiments where the dissolved oxygen remained above 55 %.

Other authors have demonstrated the positive effect of increasing stirring rates from 400 rpm to 800 rpm (Rywińska *et al.*, 2012) and to 1000 rpm (Crolla and Kennedy, 2004) on citric acid production, but did not calculate or estimate k_La.

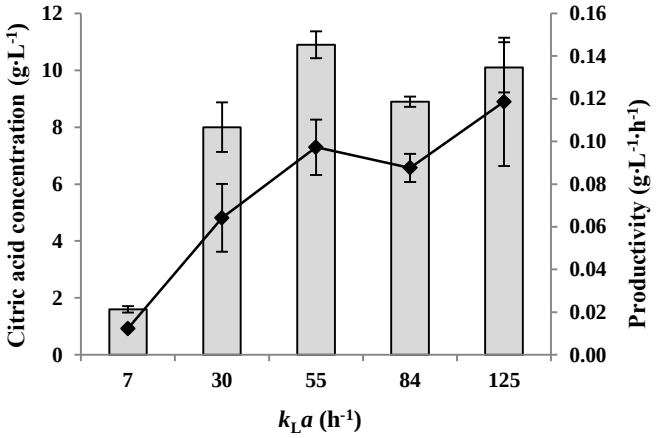

Figure 4.5 Effect of k_La on citric acid concentration (bars) and maximum productivity (dots). Citric acid productivity was calculated by the ratio between the maximum citric acid concentration obtained in each experiment and respective time. The error bars represent the standard deviation of two independent replicates.

The small amount of citric acid produced at lower k_La value can be due to variations on the activity of important enzymes involved on citric acid production. Some authors observed a decrease in citric acid production under low aeration conditions, which was associated with a decrease in the activity of enzymes involved in tricarboxylic acid cycle and glyoxylate cycle (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2003). At low aeration conditions, a reduced activity of citrate synthase, aconitate hydrate and NAD^+-dependent isocitrate dehydrogenase was observed. Additionally, an increase on the ATP-citrate lyase activity, responsible to the cleavage of citrate, was noticed. Moreover, the activity of isocitrate lyase and malate synthase, which are enzymes from glyoxylate cycle and are involved in the cleavage of isocitrate to succinate and malate, decreased under low oxygen concentration conditions. This pathway presents the major source of succinate and malate to mitochondria during intensive citric acid production. With the reduction of these enzymes activity from glyoxylate cycle, the increase of ATP-citrate lyase activity provides an alternative source for mitochondrial activity, not allowing an accumulation of citric acid (Finogenova *et al.*, 2002).

Above a k_La of 55 h^{-1}, no differences were observed in citric acid concentration and productivity. Probably, the highest stirring rates imposed a hydrodynamic stress to the cells that might affect cell morphology and viability and, consequently, yeast metabolism. Changes in

morphology of *Y. lipolytica* cells growing in high stirring and aeration rates were reported by Braga *et al.* (2015).

Among the experimental conditions studied, the highest specific growth rate was obtained in the assays carried out with a k_La value equal to 30 h^{-1} (Table 4.2). Above this value, no significant differences were observed. Moreover, the highest biomass yield was attained with the lowest value of k_La. Also Crolla and Kennedy (2004) demonstrated that increasing stirring rate from 400 rpm to 900 rpm (with constant aeration rate of 1 vvm) did not affect the *Candida lipolytica* cells concentration, but the lower stirring rates resulted in higher biomass yield. The highest specific glycerol consumption rate (q_S) and citric acid yield ($Y_{CA/S}$) were obtained in the experiments performed with a k_La equal to 55 h^{-1}. A 2.7-fold and 4.4-fold improvement in glycerol consumption rate and citric acid yield, respectively, was obtained increasing the k_La from 7 h^{-1} to 55 h^{-1}.

Table 4.2 Effect of k_La (h^{-1}) on maximum specific growth rate (μ), specific consumption rate (q_S), biomass yield ($Y_{X/S}$), citric acid yield ($Y_{CA/S}$) and isocitric/citric acid ratio (ICA/CA) during batch cultures of *Y. lipolytica* W29. Data are presented as average and standard deviation of two independent experiments.

	k_La (h^{-1})				
	7	**30**	**55**	**84**	**125**
μ (h^{-1})	0.06 ± 0.01	0.092 ± 0.004	0.09 ± 0.01	0.085 ± 0.005	0.085 ± 0.006
q_S ($g \cdot L^{-1} \cdot h$)	0.24 ± 0.05	0.51 ± 0.02	0.65 ± 0.24	0.68 ± 0.32	0.61 ± 0.15
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.26 ± 0.01	0.18 ± 0.01	0.14 ± 0.04	0.14 ± 0.08	0.14 ± 0.03
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.07 ± 0.00	0.17 ± 0.01	0.31 ± 0.11	0.23 ± 0.04	0.27 ± 0.06
ICA/CA ($g \cdot g^{-1}$)	0.21 ± 0.02	0.16 ± 0.03	0.14 ± 0.05	0.11 ± 0.01	0.10 ± 0.05

In addition to the influence on citric acid production, k_La also affected the isocitric/citric acid ratio. A considerably decrease (2.1-fold) of this parameter was attained raising the k_La from 7 h^{-1} to 125 h^{-1}. Other studies described that isocitric acid percentage decreased with an increase of stirring or aeration rates (Rywińska *et al.*, 2012). This result is particularly important for the downstream process, diminishing the global cost of citric acid purification.

4.3.3 Effect of controlled dissolved oxygen on citric acid production

Dissolved oxygen concentration in the production medium is an operational parameter that can influence the bioprocess overall performance. The maintenance of an adequate oxygen concentration through all the time is a challenge and a crucial step to maximize microbial growth and metabolites production.

Considering the oxygen profiles obtained in the previous assays and discussed above, several experiments were performed with constant dissolved oxygen concentrations and the values of 20 %, 40 % and 60 % were chosen. These values of DO were controlled by manipulating the stirring and aeration rates, through a cascade control mode. The concentrations were selected taking into account the DO profiles obtained from the k_La assays: (a) 60 % was, approximately, the value at which the dissolved oxygen stabilized in the k_La condition with higher concentration of citric acid; (b) 20 % was the lowest dissolved oxygen concentration (different of 0 %) obtained during citric acid production; and (c) 40 % is an intermediate value.

Although *Y. lipolytica* is a strictly aerobic microorganism, no differences were found in the growth profiles and cell density reached with increased DO concentrations in the medium (Figure 4.6a). Also, glycerol consumption had the same behavior for all conditions tested (Figure 4.6b). In contrast, an enhancement of citric acid production was obtained with the raise of DO concentration (Figure 4.6c). A 40 % and 60 % improvement in citric acid concentration was attained by increasing the DO concentration in the medium from 20 % to 40 % and to 60 %, respectively (Figure 4.7). Additionally, the maximum productivity was positively affected by the raise of DO concentration up to 60 % and a 30 % improvement was obtained compared to the assays carried out at 20 % DO.

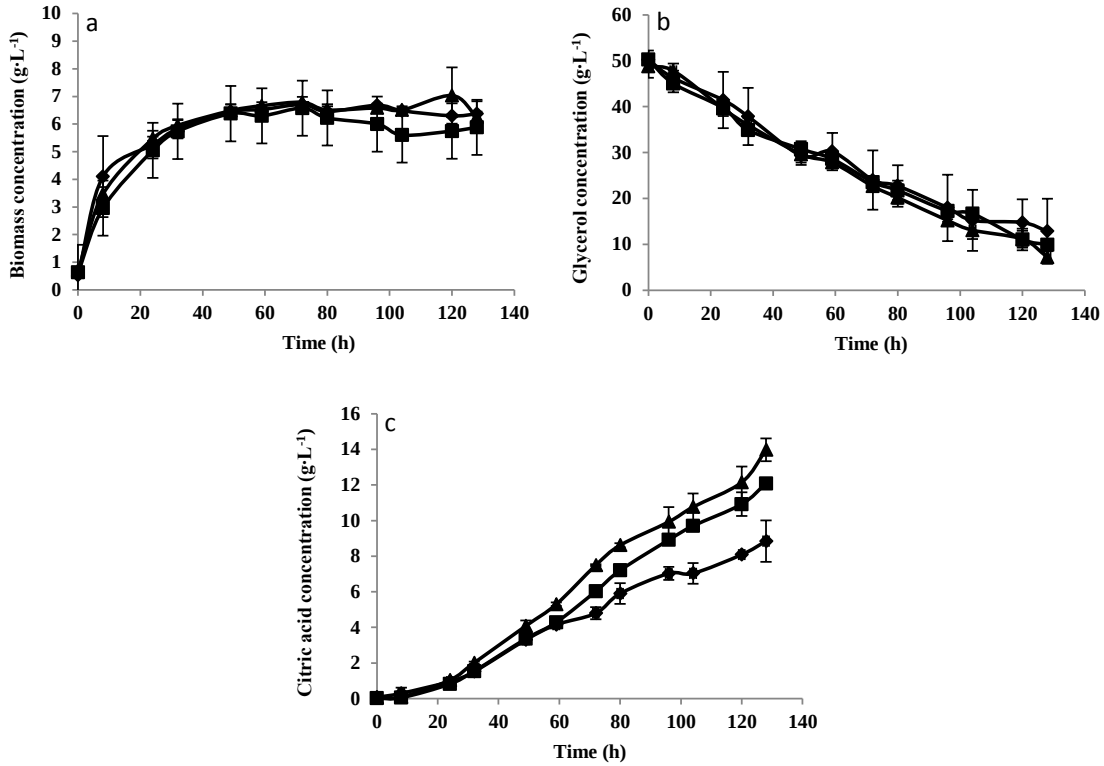

Figure 4.6 Biomass concentration (a) crude glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 with different dissolved oxygen concentrations (%): 20 (♦), 40 (■), 60 (▲). The error bars represent the standard deviation of two independent replicates.

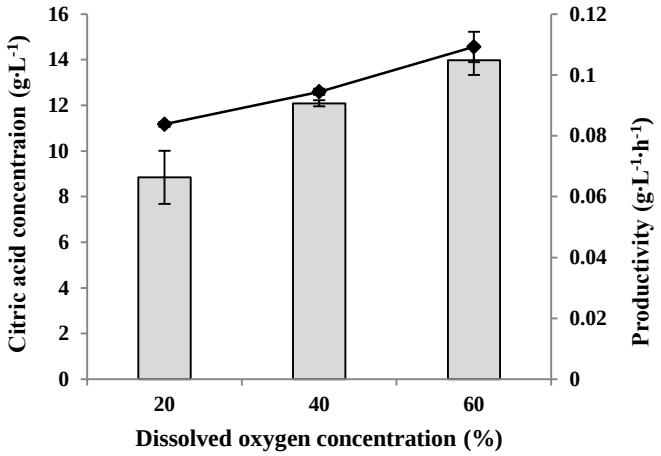

Figure 4.7 Effect of dissolved oxygen (%) on citric acid concentration (bars) and maximum productivity (dots). Citric acid productivity was calculated by the ratio between the maximum citric acid concentration obtained in each experiment and respective time. The error bars represent the standard deviation of two independent replicates.

Independently the DO concentration in the medium, no differences were observed in specific growth rate, biomass yield and glycerol specific consumption rate (Table 4.3). However, a 60 % improvement in citric acid yield was attained in the experiments with 60 % of DO compared to the assays carried out at 20 %.

Table 4.3 Effect of dissolved oxygen concentration (%) on maximum specific growth rate (μ), specific consumption rate (q_S), biomass yield ($Y_{X/S}$) and citric acid yield ($Y_{CA/S}$) during bath culture of *Y. lipolytica* W29. Data are presented as average and standard deviation of two independent experiments.

	Dissolved oxygen concentration (%)		
	20	40	60
μ (h^{-1})	0.085 ± 0.002	0.079 ± 0.006	0.077 ± 0.000
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.16 ± 0.04	0.13 ± 0.01	0.15 ± 0.02
q_S ($g \cdot g^{-1} \cdot h^{-1}$)	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.235 ± 0.006	0.299 ± 0.005	0.37 ± 0.04

These results are in accordance with previous works published by Finogenova *et al*. (2002) and Kamzolova *et al*. (2003), which reported the increase of citric acid production, in batch and continuous cultures of *Y. lipolytica*, when the DO concentration in the medium was equal to 60 %. However, Anastassiadis and Rehm (2006a) observed that the maximum citric acid production by *Candida oleophila*, in continuous mode, was achieved with an DO of 20 %.

Comparing the results obtained in the experiments carried out with constant k_La and controlled dissolved oxygen, it was observed that citric acid concentrations obtained using controlled DO (20 % and 60 %) was similar to those attained in constant k_La experiments of 30 h^{-1} and 55 h^{-1}, respectively.

Considering the small differences in citric acid production obtained in the two operational approaches discussed above, and from an industrial perspective, operating at constant k_La will be easier and less technologically demanding. Moreover, in this work, when DO was controlled at 20 % and 60 %, the stirring rates needed to achieve these oxygen concentrations were higher

than those used with constant k_La of 30 h^{-1} and 55 h^{-1} (data not shown), thus representing an increase of operating costs due to power consumption. For the reasons explained above, k_La was proven to be an adequate parameter of optimization of the bioprocess of citric acid production from glycerol by *Y. lipolytica* W29.

4.4 CONCLUSIONS

Citric acid production by *Y. lipolytica* W29, from crude glycerol, was evaluated considering the effect of oxygen using two different strategies: constant k_La and constant dissolved oxygen concentration. An empirical correlation to predict k_La value as a function of operating conditions (agitation and aeration rates), as well of cellular density was established. The increase of k_La resulted in an increase of citric acid production and a decrease of isocitric/citric acid ratio. The maximum citric acid concentration was achieved with an intermediate value of k_La (55 h^{-1}). The raise of dissolved oxygen concentration led to an increase of citric acid yield and productivity, achieved either by controlling dissolved oxygen concentration at constant values or by increasing stirring and aeration rates to give adequate k_La values. Comparing both strategies and considering an industrial implementation of the process, constant k_La appears to be a more economically attractive approach. This work demonstrated the importance of k_La in citric acid production by *Y. lipolytica* from crude glycerol. The correlation proposed herewith will be very useful for further work on the development of strategies for the optimization and scale-up of this bioprocess.

5 USE OF PRESSURIZED AND AIRLIFT BIOREACTORS FOR CITRIC ACID PRODUCTION BY *YARROWIA LIPOLYTICA* FROM CRUDE GLYCEROL

Citric acid production is generally carried out in an aqueous medium in stirred tank reactors (STR), where the solubility of oxygen is low and the oxygen demand of microbial cultures is high. Thus, providing adequate oxygen mass transfer rate (OTR) from the gas into the aqueous culture medium is a main challenge of bioreactor selection and operation. In this study, citric acid production by *Yarrowia lipolytica* W29 from crude glycerol, in batch cultures, was performed in two non-conventional bioreactors, normally associated to high mass transfer efficiency: a pressurized and an airlift bioreactor. Increased OTR's were obtained by raising the total air pressure in the pressurized stirred tank bioreactor or by increasing the aeration rate in the airlift bioreactor. An improvement of 40 % in maximum citric acid concentration and yield was obtained raising the air pressure from 1 bar to 2 bar, whereas in the airlift bioreactor, a 30 % improvement was attained by increasing the aeration rate from 1 vvm to 1.5 vvm. Both bioreactor types can be successfully applied for citric acid production process using alternative ways of improving OTR other than increasing power input, leading to important operating costs savings.

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5.1 INTRODUCTION

Citric acid production by *Yarrowia lipolytica* is an aerobic process, thus oxygen is a crucial factor for maximization of microbial growth and product formation (Workman *et al.*, 2013), this was also confirmed by the results presented in previous chapters. The oxygen mass transfer from gas phase to the liquid medium and the amount of oxygen available to cells can directly affect the quantity and the type of organic acids produced (Okoshi *et al.*, 1987; Finogenova *et al.*, 1991; Rywińska *et al.*, 2012; Kamzolova *et al.*, 2013).

The most common type of bioreactor used in citric acid production by *Y. lipolytica* is the stirred tank reactor (STR). A few disadvantages have been associated with traditional stirred tanks: (a) wide variation of shear forces inside the reactor, once the energy required to move the fluid is introduced into a single point of the reactor, which results in a higher dissipation near the stirrer and a decrease towards the walls (Merchuk, 1990); (b) due to a low oxygen mass transfer coefficient, high stirring rates are required to achieve enough oxygen mass transfer; (c) the high mechanical power input usually results in overheating; (d) the increase of mechanical power input generates high shear stress that can damage (Ohta *et al.*, 1995) or change the cells morphology (Braga *et al.*, 2015); (e) due to its complexity, STR bioreactors are more expensive, require higher maintenance costs and are less robust than other types of reactors (Braga *et al.*, 2015). Taking into account these negative aspects of traditional STR bioreactors, and mostly the limitation of oxygen mass transfer that can occur at atmospheric pressure, other alternatives should be considered, such as pressurized and airlift bioreactors (Vial *et al.*, 2002; Knoll *et al.*, 2005; Lopes *et al.*, 2014a).

Pressurized reactors are of great interest to enhance the oxygen mass transfer from the gas phase to the liquid medium (Lopes et al., 2013). In these bioreactors, the enhancement of oxygen mass transfer rate (OTR) is achieved by the increase of total air pressure, and consequently of oxygen partial pressure, leading to the raise of oxygen solubility (Lopes *et al.*, 2014a). High pressure reactors and the associated technologies are broadly used in chemical industry. The high mass transfer capacity and its cost efficiency opens new perspectives to adapt these technologies to microbial cultures (Knoll *et al.*, 2005). Published studies have already proved that pressurized bioreactors could be successfully applied to microorganisms cultivation (reviewed by Lopes *et al.* (2014a)). Several authors have demonstrated the applicability of increased air pressure (up to 15

bar) for biomass production (Belo *et al.*, 2003) and metabolites secretion enhancement, such as extracellular lipase (Lopes *et al.*, 2008), homologous β-galactosidase (Pinheiro *et al.*, 2003) and heterologous proteins (Lopes *et al.*, 2014b). Moreover, it was shown that, when high OTR values are needed, the raise of air pressure could be a way of improving OTR, with energy cost efficiencies acceptable for industrial application (Knoll *et al.*, 2005).

Airlift bioreactors are pneumatically agitated with unique hydrodynamic characteristics and often employed in bioprocesses where gas-liquid mass transfer is an important parameter (Merchuk *et al.*, 1994). This type of bioreactor presents some advantages compared with conventional STR: (a) uniform shear distribution; (b) high liquid velocity and intensity of turbulence, that allows an increase of heat transfer capacity, mass transfer rate and good mixing properties at low energy consumption; (c) both aeration and agitation of production medium are due to the gas phase; and (d) low shear stress (Vial *et al.*, 2002). Studies conducted in airlift bioreactors have shown the great potential of this type of bioreactor for the development of bioprocesses based in *Y. lipolytica*, namely the biotransformation of methyl ricinoleate and castor oil into lactones (Escamilla-García *et al.*, 2014; Braga *et al.*, 2015) and citric acid production with immobilized cells (Kautola *et al.*, 1991; Rymowicz *et al.*, 1993).

In this work, the production of citric acid in batch cultures by *Y. lipolytica* W29 from crude glycerol was studied in two bioreactor types – pressurized STR and airlift bioreactors. To best knowledge, this is the first time that citric acid production is performed in a pressurized bioreactor under increased air pressure or in an airlift bioreactor with suspended cells. Thus, in this work, the effect of increased air pressure (in pressurized reactor) and aeration rate (in airlift reactor) on citric acid production was evaluated.

5.2 MATERIAL AND METHODS

5.2.1 Yeast strain and culture conditions

Y. lipolytica W29 (ATCC 20460) was maintained in YPDA medium (described in chapter 3.2.1) at 4 °C for a maximum of 2 weeks.

Cells of *Y. lipolytica* were pre-grown in 500 mL Erlenmeyer flasks with 200 mL of medium, composed by pure glycerol 20 $g \cdot L^{-1}$, peptone 20 $g \cdot L^{-1}$ and yeast extract 10 $g \cdot L^{-1}$, for 18 h at 27 °C in an incubator shaker at 200 rpm.

5.2.2 Pressurized bioreactor

In order to evaluate the effect of increased air pressure on citric acid production, several batch cultures were performed in a stainless stirred tank bioreactor (PARR 4563, Parr Instruments, USA) with 600 mL of capacity and a working volume of 400 mL (Figure 5.1). The production medium had the following composition ($g \cdot L^{-1}$): crude glycerol 20; yeast extract 0.5; $MgSO_4 \cdot H_2O$ 1.5; KH_2PO4 24; Na_2HPO_4 2; salts solution ($CaCl_2$ 0.75; $FeCl_3 \cdot 6H_2O$ 0.75; $ZnSO_4 \cdot 7H_2O$ 0.1; $MnSO_4 \cdot H_2O$ 0.3). Batch cultures started with 0.5 $g \cdot L^{-1}$ of cells and were performed at 27 °C, 400 rpm and initial pH of 5. Compressed air was continuously sparged into the culture at an aeration rate of 1 vvm (under standard conditions of temperature and pressure). Reactor pressure was set by manipulating inlet air pressure and the regulatory valve in the exit gas line. The bioreactor was equipped with a pressure transducer (Parr 4842, Parr Instruments, USA) to monitor the total internal pressure. The values of total air pressure studied were 1 bar, 2 bar and 4 bar.

Figure 5.1 Pressurized bioreactor Parr (PARR 4563, Parr Instruments, USA).

5.2.3 Airlift bioreactor

Several experiments were carried out in an airlift bioreactor, varying the aeration rate, and its effect on citric acid production was assessed. Airlift bioreactor (Figure 5.2) was constructed in glass with a working volume of 4 L and 0.7 m of inside diameter. The riser-tube had 0.37 m of height and an inside diameter of 0.032 m. Air was used as gas stream in the gas-liquid contactor and it was fed at the bottom of the bioreactor using a five holes sparger. Dissolved oxygen (DO) concentration in the medium was measured with a polarographic-membrane probe and monitored with a computer interface (CIODAS08JR, Computer Boards, USA) using the LABtech Notebook software (Datalab Solution, USA).

After pre-growth overnight, yeast cells were collected and transferred to the production medium composed by ($g \cdot L^{-1}$): crude glycerol 50; Yeast extract 0.5; $MgSO_4 \cdot H_2O$ 1.5; KH_2PO4 6; Na_2HPO_4 0.5; salts solution ($CaCl_2$ 0.75; $FeCl_3 \cdot 6H_2O$ 0.75; $ZnSO_4 \cdot 7H_2O$ 0.1; $MnSO_4 \cdot H_2O$ 0.3). The medium was inoculated with 1.5 $g \cdot L^{-1}$ of cells and the assays were performed at 27 °C and pH of 5.0.

Figure 5.2 Airlift bioreactor with production medium.

5.2.4 OTR calculation

The OTR values estimation for pressurized bioreactor, that measures the maximum possible value of OTR at operating conditions used, were obtained by the sulfite oxidation method as previously described (Lopes et al., 2013).

Static gassing-out technique was used to determine OTR in airlift bioreactor (Wise, 1951) as described in chapter 4.2.1.

5.2.5 Analytical methods

Samples were periodically collected to measure biomass concentration, glycerol consumption and citric production. The samples analysis was performed as described in chapter 3.2.3.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of operating conditions on OTR

For a specific bioreactor and culture medium, the increase of aeration rate, stirring rate and oxygen solubility in the medium results in an OTR enhancement. Increasing air pressure from 1 bar to 4 bar led to a 2-fold improvement in OTR (Figure 5.3). This result is in accordance with Henry's law, in which air pressure raise increases the oxygen solubility in the medium and consequently improves OTR (Lopes *et al.*, 2014a). In the airlift bioreactor the airflow rate promotes aeration and stirring, thus increasing OTR(Vial *et al.*, 2002) The increase of aeration rate from 1 vvm to 2 vvm led to a 2.5-fold OTR improvement (Figure 5.3).

In the pressurized bioreactor OTR values were higher than in the airlift bioreactor. This shows that air pressure increase may be used to obtain high OTR values. Nevertheless, it must be stressed that OTR obtained by sulfite method may be overestimated, mainly due to the physicochemical properties differences between aqueous sulfite solution and fermentation medium used in the static method, that affects bubbles coalescence and consequently the interfacial area for mass transfer (Belo *et al.*, 2000).

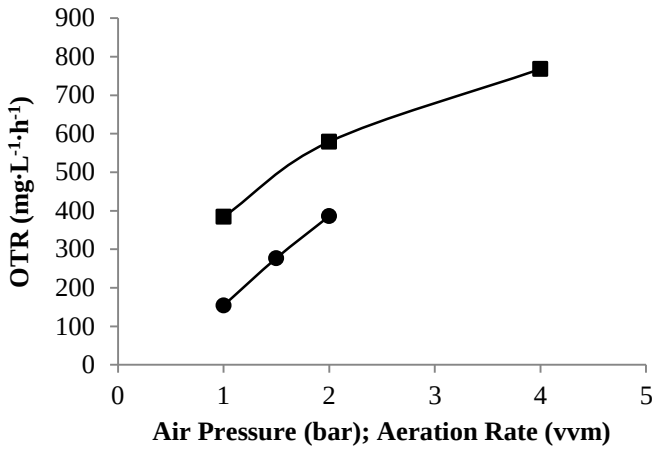

Figure 5.3 Oxygen mass transfer rate (OTR) variation under different operating conditions in: Pressurized bioreactor (■) – OTR *vs* air pressure; Airlift bioreactor (●) – OTR *vs* aeration rate.

5.3.2 Effect of increased air pressure on citric acid production

To study the effect of increased air pressure on citric acid production by *Y. lipolytica* W29, several assays were conducted in a pressurized bioreactor under total air pressure of 1 bar (equivalent to atmospheric pressure), 2 bar and 4 bar. The time course of cellular growth, glycerol consumption and citric acid production at different values of air pressure are presented in Figure 5.4. The raise of air pressure had no significant effect on cellular growth (Figure 5.4a), thus no inhibitory effects were observed under air pressure of 4 bar as compared to 1 bar. Lopes *et al.* (2009) reported an enhancement of cellular growth of *Y. lipolytica* W29 and an increase of carbon source consumption rate under 6 bar of air pressure. However, the study was carried out in a rich medium (without nitrogen limitation) and glucose as carbon source. The maximum citric acid concentration (Figure 5.4b) was obtained in the experiments conducted at 2 bar of air pressure and no significant differences were obtained in the final citric acid concentration attained under 4 bar and atmospheric pressure (Figure 5.4c). However, it seems that there was a slight augmentation of crude glycerol consumption in the experiments carried out at 4 bar of air pressure (Figure 5.4b).

The raise of air pressure from 1 bar to 2 bar led to a 40 % improvement on citric acid concentration and above this value of air pressure a decrease was observed. The increase of citric

acid production with the raise of air pressure up to 2 bar can be due to the activity enhancement of several enzymes of the tricarboxylic acid and glyoxylate cycles, which are involved in the citric acid production. The raise of air pressure led to an increase of oxygen solubility in the culture, that may enhance some enzymes activity, such as citrate synthase, isocitrate lyase, aconitate hydrate and NAD^+-dependent isocitrate dehydrogenase, according with previous reported works performed at atmospheric pressure (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2003). The slight decrease of citric acid concentration obtained at 4 bar of air pressure may probably be due to a shift in yeast metabolism, mainly in the tricarboxylic acid cycle. Aguedo *et al.* (2005) also reported a change in the metabolic pathway of γ-decalactone production when cells of *Y. lipolytica* W29 were growing under 10 bar of total air pressure.

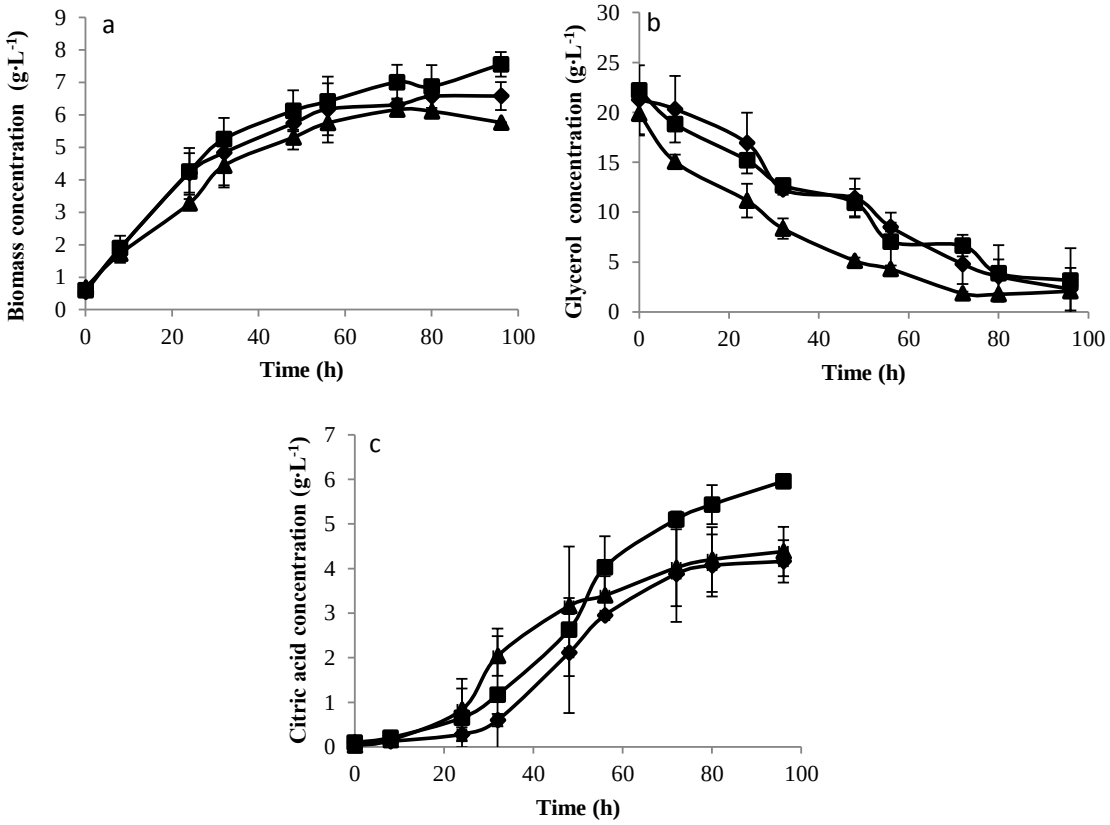

Figure 5.4 Biomass concentration (a) glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 at 1 bar (♦), 2 bar (■) and 4 bar (▲) of air pressure. The error bars represent the standard deviation of two independent replicates.

Among the values of air pressure tested, the highest specific growth rate and citric acid yield were obtained under 2 bar of air pressure (Table 5.1). A slight increase of maximum specific citric acid productivity (q_{CA}) with pressure was observed but still statistically insignificant. No significant differences were observed in biomass yield at different pressures in spite of the slight higher value observed at 2 bar. However, a positive effect on biomass yield and specific growth rate of *Y. lipolytica* W29 cultures under increased air pressure was described by other authors (Aguedo *et al.*, 2005; Lopes *et al.*, 2009). On the other hand, Lopes *et al.* (2014b) observed no effect on maximum specific growth rate, biomass yield and specific consumption rate with increased air pressure up to 5 bar in batch cultures of two recombinant *Pichia pastoris* strains. Previous reports proved that the effect of increased air pressure was dependent not only on yeast strain but also on operational conditions. The raise of air pressure in *Saccharomyces cerevisiae* batch cultures led to a decrease in biomass productivity (Pinheiro *et al.*, 1997), but had a positive effect in fed-batch cultures (Belo *et al.*, 2003).

Table 5.1 Effect of increased air pressure on maximum specific growth rate (μ), biomass yield ($Y_{X/S}$), specific consumption rate (q_S), citric acid yield ($Y_{CA/S}$) and maximum specific citric acid productivity (q_{CA}) during bath cultures of *Y. lipolytica* W29 in a pressurized bioreactor. Data are presented as average and standard deviation of two independent experiments.

	1 bar	2 bar	4 bar
μ (h^{-1})	0.064 ± 0.003	0.077 ± 0.007	0.062 ± 0.004
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.33 ± 0.08	0.37 ± 0.05	0.29 ± 0.03
q_S ($g \cdot g^{-1} \cdot h^{-1}$)	0.20 ± 0.04	0.21 ± 0.01	0.22 ± 0.01
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.23 ± 0.04	0.32 ± 0.05	0.25 ± 0.05
q_{CA} ($g \cdot g^{-1} \cdot h^{-1}$)	0.009 ± 0.001	0.011 ± 0.001	0.012 ± 0.002

5.3.3 Effect of aeration rate on citric acid production in airlift bioreactor

Airlift bioreactors are used in some microbial processes due to its high oxygen transfer capacity and less shear stress imposed to the cells. There are a few studies regarding the production of citric acid by *Y. lipolytica* in airlift bioreactors but only with immobilized cells (Kautola

et al., 1991; Rymowicz *et al.*, 1993). Moreover, none of them studied the effect of aeration rate in bioprocess yield and productivity. To evaluate the effect of aeration rate on citric acid production by *Y. lipolytica* W29 from crude glycerol in an airlift batch bioreactor, several experiments were performed with three aeration rates (1 vvm, 1.5 vvm and 2 vvm).

The increase of aeration rate from 1 vvm to 2 vvm had a positive effect on cellular growth (Figure 5.5a) and a 40 % improvement in the final biomass concentration was obtained compared to 1 vvm. Crude glycerol consumption (Figure 5.5b) was slightly augmented with the increase of aeration rate from 1 vvm to 2 vvm. However, the citric acid production was only improved with the increase of aeration rate from 1 vvm to 1.5 vvm (Figure 5.5c). Above this value, a decrease in citric acid concentration was observed.

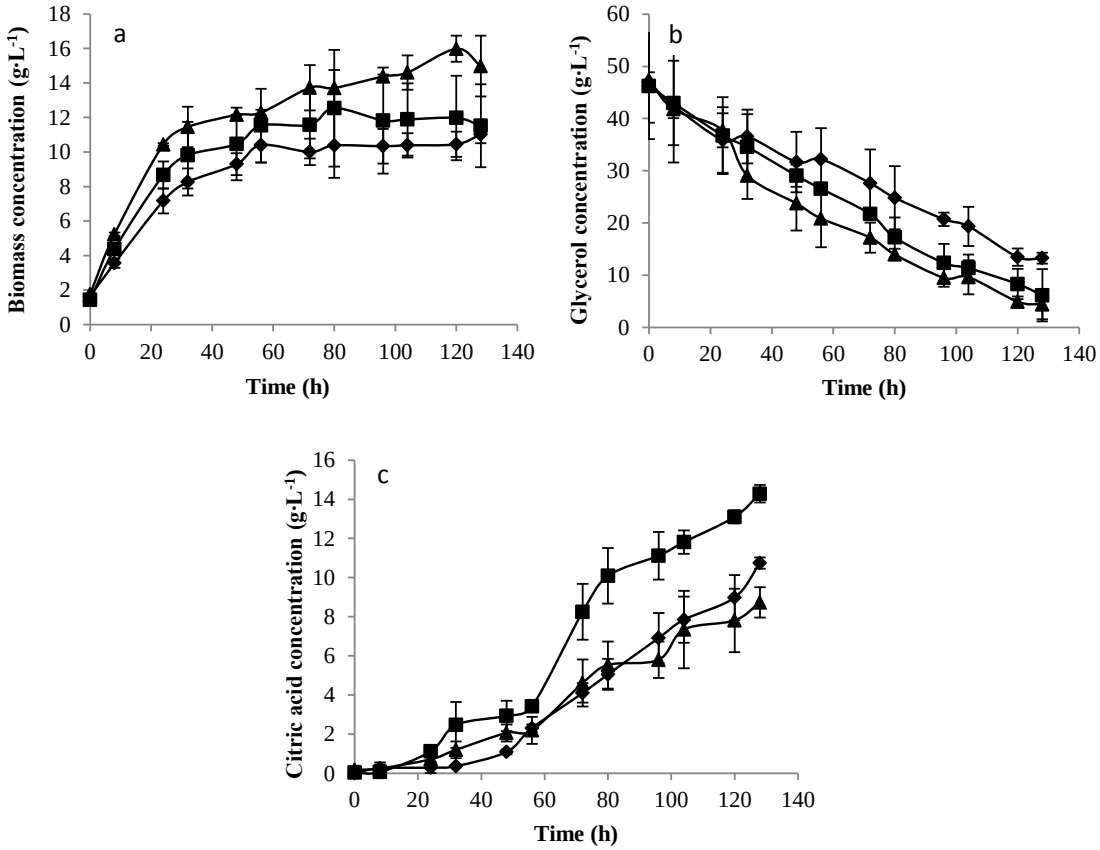

Figure 5.5 Biomass concentration (a) glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 in an airlift bioreactor at 1 vvm (♦), 1.5 vvm (■) and 2 vvm (▲). The error bars represent the standard deviation of two independent replicates.

A 30 % improvement of citric acid concentration was obtained increasing the aeration rate from 1 vvm to 1.5 vvm (Figure 5.5c). The increase of aeration rate from 1.5 vvm to 2 vvm resulted in the decrease of citric acid concentration. A similar behavior was described by Yuguo *et al.* (1999) that reported a slight decrease of citric acid production by *Aspergillus niger* in an external-loop airlift bioreactor, raising the aeration rate from 1.3 vvm to 1.4 vvm. Braga *et al.* (2015) also described a decrease on γ-decalactone maximum concentration with the increase of aeration rate in batch cultures of *Y. lipolytica* W29 performed in airlift bioreactor.

Among the aeration rates tested, the highest specific growth rate was obtained at 1.5 vvm and 2 vvm (Table 5.2). The raise of aeration rate up to 2 vvm had a clearly positive effect on biomass yield and a 30 % improvement was attained compared to 1 vvm. There was no relevant effect of aeration rate on specific consumption rate and the value of this parameter was similar for all the conditions tested. Analogous to citric acid concentration, both citric acid yield and maximum specific citric acid productivity were enhanced with the raise of aeration rate from 1 vvm to 1.5 vvm and decreased above this value.

Table 5.2 Effect of aeration rate on maximum specific growth rate (μ), biomass yield ($Y_{X/S}$), specific consumption rate (q_S), citric acid yield ($Y_{CA/S}$) and maximum specific citric acid productivity (q_{CA}) during bath culture of *Y. lipolytica* W29 in an airlift bioreactor. Data are presented as average and standard deviation of two independent experiments.

	1 vvm	1.5 vvm	2 vvm
μ (h^{-1})	0.059 ± 0.001	0.069 ± 0.005	0.070 ± 0.005
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.27 ± 0. 05	0.29 ± 0.04	0.34 ± 0.03
q_S ($g \cdot g^{-1} \cdot h^{-1}$)	0.22 ± 0.04	0.25 ± 0.02	0.21 ± 0.03
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.3 ± 0.1	0.4 ± 0 .1	0.20 ± 0.02
q_{CA} ($g \cdot g^{-1} \cdot h^{-1}$)	0.009 ± 0.001	0.012 ± 0.004	0.005 ± 0.001

As expected, according with OTR values, different dissolved oxygen profiles were observed in batch cultures of *Y. lipolytica* in the airlift bioreactor (Figure 5.6). A decrease on oxygen concentration was observed in the first hours of yeast cultivation that corresponds to exponential growth phase. This decrease was more pronounced for 1 vvm, where the completely depletion of

oxygen was observe during the first hours. The oxygen demand is lower during citric acid production phase (when the nitrogen source had been completely consumed), resulting in an increase of oxygen concentration in the medium (Rane and Sims, 1994; Wentworth and Cooper, 1996; Rywińska *et al.*, 2012). In the experiments carried out at 1 vvm of aeration rate, the DO concentration dropped to zero in the first hours and stabilized around 10 % during the citric acid production. For the other aeration conditions, the oxygen concentration in the medium never reached zero and stabilized around 35 % and 60 % for 1.5 vvm and 2 vvm, respectively.

Some authors reported a decrease on the activity of some enzymes involved in the citric acid production at DO concentration close to 5 %, which leads to a decrease in citric acid concentration (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2003). This observation can explain the lower citric acid production obtained at 1 vvm. An optimal DO concentration around 50 % to 60 % was reported in the literature for citric acid production processes (Okoshi *et al.*, 1987; Anastassiadis and Rehm, 2006b; Rywińska *et al.*, 2012). The maximum citric acid concentration in the airlift bioreactor was attained in the experiments performed at 1.5 vvm, in which the DO remained near to 35 % during the citric acid production; above this value (DO around to 55 % - 60 %), a citric acid concentration decrease was observed. Anastassiadis and Rehm (2006b) reported that for DO concentrations lower or higher than 20 % the citric acid production decreased in *Candida oleophila* ATCC 20177 continuous cultures. The authors suggested a "kind of Crabtree effect", since high glycolytic flow rate was attained, simulating an anaerobic glycolytic pathway under aerobic conditions (Anastassiadis and Rehm, 2006b).

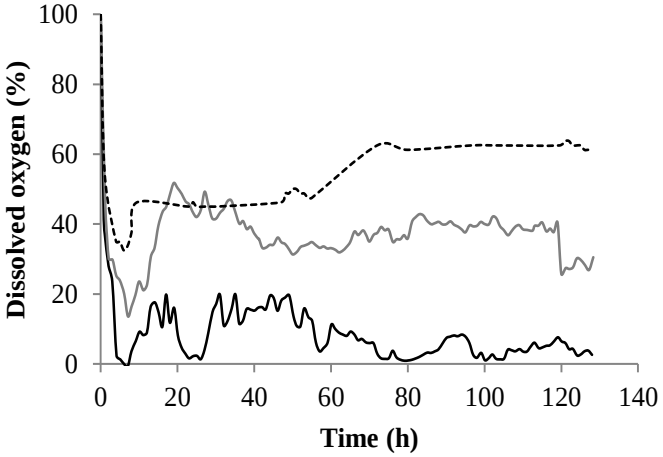

Figure 5.6 Dissolved oxygen concentration profiles during citric acid production in batch cultures of *Y. lipolytica* W29 in an airlift bioreactor at different aeration rates: 1 vvm (black line); 1.5 vvm (grey line); 2 vvm (dashed line).

In this work, maximum specific citric acid productivity of 0.012 $g \cdot g^{-1} \cdot h^{-1}$ was reached in both bioreactors by oxygenation conditions improvement. A 30 % enhancement on maximum specific citric acid productivity resulted from the increase of air pressure from 1 bar to 4 bar in the pressurized bioreactor, either from aeration rate increase from 1 vvm to 1.5 vvm in the airlift bioreactor.

Using alternative ways of improving OTR, both bioreactor types can be successfully implemented in citric acid production process with important operating costs savings. Particularly, the use of pressurized bioreactors will also reduce the needs of high aeration rates that present limitations of causing turbulence and foam problems in bioreactors operation.

5.4 CONCLUSIONS

Citric acid production from crude glycerol by *Y. lipolytica* W29 batch cultures was studied in pressurized and airlift bioreactors. No cellular growth inhibition was observed raising the total air pressure from 1 bar to 4 bar, but maximum citric acid concentration and yield were obtained under 2 bar of air pressure. In airlift bioreactor, the aeration rate increase up to 2 vvm had a clear positive effect on final biomass concentration, but the maximum citric acid concentration and maximum specific citric acid productivity was attained at 1.5 vvm. These results clearly show that oxygenation is a crucial optimization parameter in different bioreactor types. Nevertheless, in the range of conditions tested in this work, it was possible to reach a similar maximum specific citric acid productivity in both bioreactors, proving the applicability of both bioreactors type in the citric acid production process using *Y. lipolytica* cultures and crude glycerol as substrate. Both alternatives of OTR improvement will lead to important operating costs savings by the reduction of power input consumption. Additionally, pressurized bioreactors will also allow the reduction of foam and hydrodynamic stress caused by the use of high aeration rates.

6 IMPROVEMENT OF *YARROWIA LIPOLYTICA* W29 BY MUTAGENESIS FOR CITRIC ACID PRODUCTION FROM CRUDE GLYCEROL

The simultaneous production of the isomer isocitric acid is the major problem of using *Yarrowia lipolytica* for citric acid production. Isocitric acid lower buffer capacity and chelating ability can affect citric acid crystallization, being a significant problem during the purification process. Thus, in order to obtain improved strains with reduced isocitric/citric acid ratio and/or enhanced citric acid production, *Y. lipolytica* W29 (ATCC 20460) was treated with ultraviolet (UV)-irradiation and/or ethyl methane sulfonate (EMS). Acetate-negative mutants, strains that did not grow or displayed a reduced/retarded growth on acetate, were selected for screening citric acid production profile. Thirty seven mutant strains were selected and citric acid production from crude glycerol for each strain was accessed in flask assays. From the strains tested, *Y. lipolytica* UV-75 and UV/EMS-10 presented the most interesting results. 4 times lower isocitric/citric ratio was observed for the mutant *Y. lipolytica* UV-75. The mutant UV/EMS-10 strain presented a 60 % and 90 % increase of citric acid concentration and yield, respectively. Batch cultures in bioreactor were performed to compare the biomass growth, citric and isocitric acid production of these two strains with the parental one. *Y. lipolytica* UV/EMS-10 presented a 76 % enhancement of citric acid concentration and 2.2-fold of citric acid yield comparing with the strain W29.

6.1 INTRODUCTION

Citric acid is traditionally produced by the filamentous fungi *Aspergillus niger,* although the use of yeast to produce this organic acid has been studied. Between other advantages, the possibility of use agro-industrial waste and byproducts as carbon source (Gonçalves *et al.*, 2009; Chatzifragkou and Papanikolaou, 2012; Karasu-Yalcin, 2012), like crude glycerol from biodiesel industry makes *Y. lipolytica* a very interesting citric acid producer. However, the simultaneous production of isocitric acid represents a major drawback in citric acid production by *Y. lipolytica*. Due to it lower buffer capacity and chelating ability, isocitric acid can affect crystallization of citric acid causing problems in purification process (Holz *et al.*, 2009). In *Y. lipolytica* cultures, the isocitric/citric acid ratio depends strongly on the carbon source used and culture conditions. *Y. lipolytica* growing on hydrophobic carbon sources produces higher amounts of isocitric acid comparing with yeasts growing on glucose or glycerol (Fickers, *et al.*, 2005). Selection of best culture condition can reduce the amount of isocitric acid produced, but this reduction is still not enough. Thus, an improvement of *Y. lipolytica* strains by mutagenesis can be performed in order to reduce isocitric/citric acid ratio and enhance citric acid production (Finogenova *et al.*, 2008; Rywińska *et al.*, 2010; Karasu-Yalcin, 2012). The most employed technique used to improve *Y. lipolytica* strains have been mutation using chemical or physical mutagens (Finogenova *et al.*, 2008; Karasu-Yalcin, 2012). The mutagens usually used are UV irradiation or γ-irradiation and different chemical mutagens, also a combination of both mutagens can be applied. After the treatment, the colonies which cannot grow or present a reduced/retarded growth in medium with acetate has carbon source are selected. In fact, *Y. lipolytica* strains can efficiently grow acetate as sole carbon source, thus the loss of the ability to grow on acetate is related to various abnormalities in glyoxylate cycle, which has an important role in citric acid metabolism (Barth and Gaillardin, 1997; Finogenova *et al.*, 2008).

The aim of this work was to isolate mutant strains from *Y. lipolytica* W29, with higher capacity of producing citric acid and/or strains with lower isocitric/citric acid ratio from crude glycerol. The parental strains was submitted to a treated with Ultraviolet (UV) irradiation (physical mutagen) and ethyl methane sulfonate (EMS) (chemical mutagen). The acetate-negative (ace^-) strains isolated were screened to access the citric acid capability of each strain. Finally, the strains with the better phenotype were used in batch culture studies in a lab-scale stirred tank bioreactor (STR).

6.2 MATERIAL AND METHODS

6.2.1 Yeast strains

Yarrowia lipolytica W29 (ATCC 20460) and the isolated mutants were maintained on YPDA medium (described in chapter 3.2.1) and kept at 4 °C.

6.2.2 Mutagenesis

Y. lipolytica W29 (parental strain) was treated with 2 mutagenic agents: a physical – UV-irradiation and a chemical – ethyl methane sulfate (EMS). Yeast was pre-grown for 18 h in 100 mL Erlenmeyer flask filled with 50 mL of YPD liquid medium, at 27 °C and 200 rpm. A suspension with $2x10^8$ cells·mL^{-1} was centrifuged for 10 min at 4 °C and 4000 rpm, washed twice with sterile NaCl 0.9 % (w/v) and resuspended in sterile sodium phosphate buffer 0.1 M (pH 7.0). This cell suspension was submitted to mutagenic agents.

In UV treatment, *Y. lipolytica* W29 cell suspensions were transferred to Petri dishes and exposed to an UV lamp (germicidal lamp (2 x 8 W) at 12 cm of distance and 245 nm of wavelength) during different times: 5 min, 15 min, 30 min and 60 min. After the exposure to UV, different dilutions of this cell suspension were spread onto medium yeast extract peptone glycerol agar (YPGA) medium plate, grown for 24 h to 48 h at 27 °C and kept at 4 °C. The YPGA medium composition was (g·L^{-1}): peptone 20 glycerol 20, yeast extract 10 and agar 20.

In the chemical treatment, EMS was used as mutagenic agent. Cells were incubated with different EMS concentrations (1.5 %, 3 %, 4.5 %, 6 % (v/v)) for 1 h at 27 °C and 200 rpm. After incubation, EMS action was stopped with the addition of thiosulfate 5 % (w/v). Successive dilutions were spread on YPGA plates and incubated for 24 h to 48 h at 27 °C.

A combined treatment was also performed, in which cells were exposed to both mutagens agents, as described above for each treatment. The conditions used in the combined treatment were, for UV-irradiation, 5 min or 15 min and for EMS, 3 % or 4.5 % (v/v)

6.2.3 Selection of mutants on acetate medium

After each treatment, the colonies obtained in YPGA medium were transferred to acetate medium and incubated at 27 °C for 24 h to 48 h. Yeast colonies were grown in a medium with acetate as sole carbon source, composed by ($g \cdot L^{-1}$): sodium acetate 5; KH_2PO_4 1; NH_4Cl 1; $MgSO_4 \cdot 7H_2O$ 0.5; and agar 20. The strains unable to grow or that exhibited a retarded growth on this medium, were selected for further citric acid production assessment. Strains selected were inoculated in YPGA medium, stored at 4 °C and used for citric acid production assays.

6.2.4 Evaluation of citric acid production by selected mutant strains

Ace^- mutant strain, isolated before, were pre-grown for 18 h in 500 mL Erlenmeyer flask filled with 200 mL of pure glycerol 20 $g \cdot L^{-1}$, peptone 20 $g \cdot L^{-1}$ and yeast extract 10 $g \cdot L^{-1}$ medium, at 27 °C and 200 rpm.

Cells were centrifuged and resuspended in 200 mL of production medium composed by ($g \cdot L^{-1}$): crude glycerol 50; yeast extract 0.5; $MgSO_4 \cdot H_20$ 1.5; KH_2PO_4 6; Na_2HPO_4 0.5; $CaCl_2$ 0.75; $FeCl_3 \cdot 6H_2O$ 0.75; $ZnSO_4 \cdot 7H_2O$ 0.1; $MnSO_4 \cdot H_20$ 0.3. Crude glycerol was provided by Prio Energy - Prio Biocombustíveis, SA and has the following composition (w/w): 90.4 % glycerol, 9 % water, 4.9 % NaCl and less than 0.001 % methanol and 0.5 % of organic matter (non-glycerol).

Screening assays were performed in 500 mL baffled flasks with 200 mL of production medium, at 27 °C in an incubator shaker at 200 rpm. During the experiments pH was maintained at 5.0 ± 0.5 by adding KOH 5 M.

6.2.5 Bioreactor assay

The strains that presented better results in the flask assays were selected and the citric acid production was accessed in a stirred tank bioreactor. The batch assays were carried out in a 3.7 L stirred tank bioreactor, STR (RALF PLUS SOLO, Bioengineering, Switzerland) (described at chapter 4.2.2).

Yeast cells were pre-grown overnight, centrifuged and resuspended in 1.7 L of production medium (described in 6.2.4) to an initial cell density of 0.5 $g \cdot L^{-1}$. The assays were carried out at 27 °C, 2 vvm of aeration rate and 400 rpm of stirring rate. Medium pH was maintained at 5 by addition of KOH 2 M or H_3PO_4 21 % (v/v), through Peripex peristaltic pumps (Bioengineering, Switzerland). Dissolved oxygen concentration was measured with a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA) using the BioScadaLab software.

6.2.6 Analytical methods

Samples were periodically collected to measure biomass concentration, glycerol consumption and citric and isocitric acids production. The samples analyses were performed as described in the chapter 3.2.3. The isocitric acid was quantified the same way as citric acid.

6.3 RESULTS AND DISCUSSION

6.3.1 Selection of acetate-negative strains

After all treatments with mutagenic agents (ultraviolet irradiation and EMS), 4562 colonies were isolated and growth on acetate medium was evaluated. Among all of the colonies studied only 37 did not grow or had a weak or retarded growth in acetate medium. From the 37 ace^- mutants selected, 23 strains resulted from the UV-irradiation treatment, 9 from EMS and only 5 from the combination of both treatments.

Most of *Y. lipolytica* strains are able to grow efficiently on acetate as the only carbon source. Thus, screening ace^- strains implies the selection of mutants that lost the ability of growing on acetate, which is related with abnormalities in the tricarboxylic acid (TCA) and glyoxylate cycles (Finogenova *et al.*, 2008; Karasu-Yalcin, 2012). The utilization of acetate by the yeasts has been related with the induction of glyoxylate cycle, which has an important role in citric acid metabolism. Mutants unable to metabolize acetate were blocked in the activity of acetyl-coenzyme A synthase. Acetyl-CoA is required to induce glyoxylate cycle, which is not active in the acetyl-CoA deficient mutants (Barth and Gaillardin, 1997). Mutants producers of citric acid were described as

displaying also low activity of aconitase, which converts citric acid into isocitric acid (Finogenova *et al.*, 2008).

6.3.2 Evaluation of citric acid production by selected mutant strains

After selecting the ace$^-$ strains, it is important to determine if the respective phenotypes led to an improvement of citric acid production, to a decrease of isocitric/citric acid or neither of these profiles. Citric and isocitric acid production by the 37 selected mutants were evaluated and compared with the parental strain *Y. lipolytica* W29 in batch cultures using crude glycerol, a by-product from biodiesel industry.

The mutagenic treatments successfully induced mutation that enhanced citric acid production. Some strains produced higher citric acid concentration than the parental strain (figure 6.1). The maximum citric acid concentration (Figure 6.1a) obtained by strains UV-27, UV-54, UV-31, UV/EMS-10 and EMS-UV-3 were 15 g·L^{-1}, 13 g·L^{-1}, 12 g·L^{-1}, 16 g·L^{-1} and 12 g·L^{-1}, respectively, that were higher than the value of 10 g·L^{-1} obtained for the parental strain. However, only UV/EMS-10 was considered statistically different ($p < 0.05$) from the W29 strain. This strain presented a 60 % and 90 % increase of citric acid concentration and citric acid yield (0.52 g·g^{-1}), respectively (Figure 6.1c) when compared with the parental strain. In some mutant strains the treatment resulted in a reduction on the citric acid production. Observing the figure 6.1a and 6.1c from the 27 strains tested 16 produce lower concentration of citric acid and 8 presented also lower yield. Considering the isocitric/citric ratio most of the mutants present a similar results comparing with the parental strain. However, three of the strains with lower citric acid concentrations, *Y. lipolytica* UV-72, UV-303 and EMS-72, presented a 14-, 8- and 11-fold higher isocitric/citric acid ratio, respectively (Figure 6.1b) comparing with the parental strain. On the opposite side, UV-75 strain presented a much lower ratio, isocitric/citric ration for UV-75 strain was 4 times lower than the parental strain. In spite of isocitric/citric acid ratio value being very small comparing with the parental strain, the values are not statistically different ($p > 0.05$).

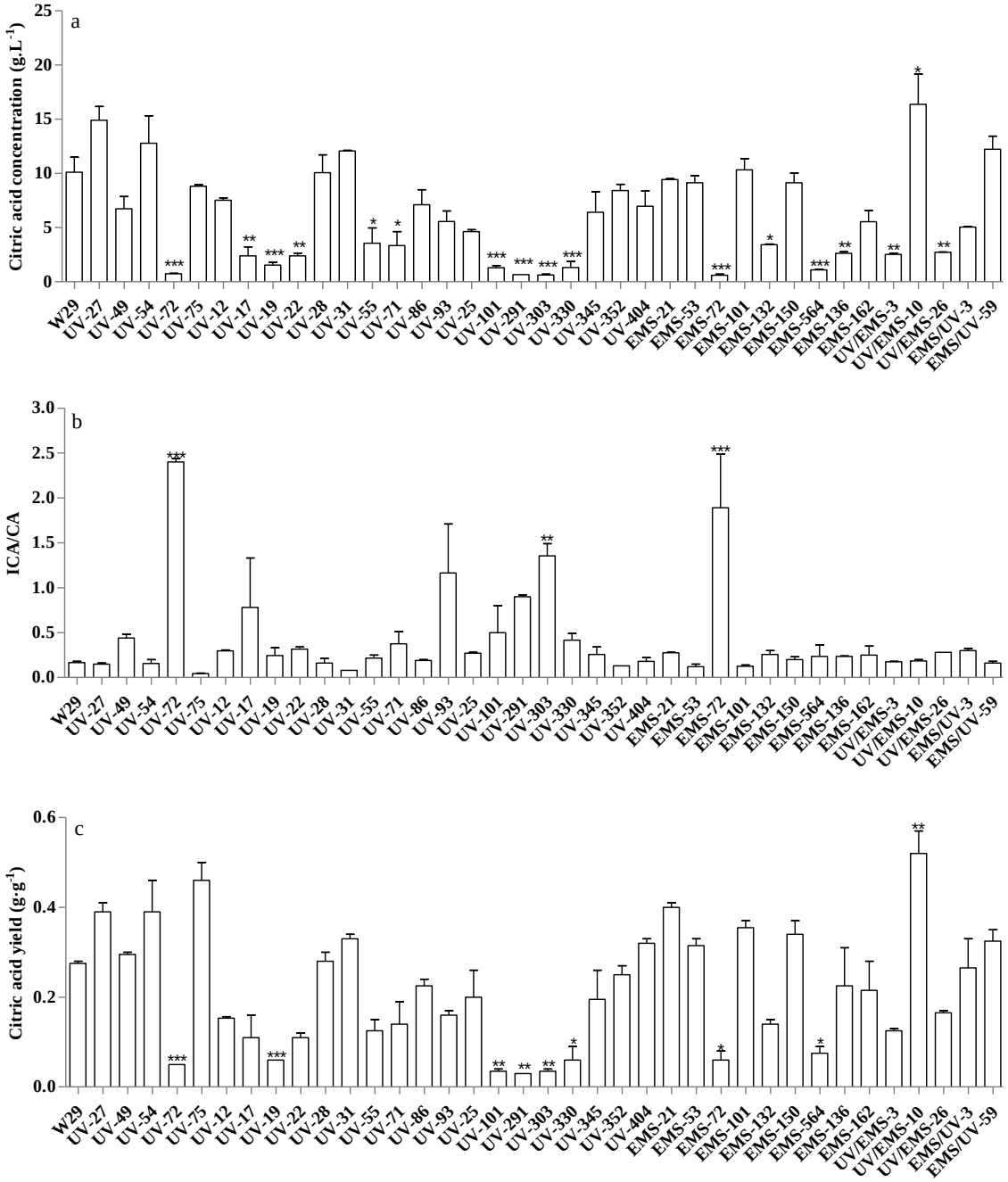

Figure 6.1 Citric acid concentration (a), ICA/CA - isocitric/citric acid ratio (b) and citric acid yield (c) for batch cultures of *Y. lipolytica* W29 and mutant strains. The values are presented as average and standard deviation of two independent experiments. The symbol (*) above the bars represents results that are statistically different from parental strain: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

The selection of strains that do not grow or have reduced/retarded growth in acetate medium is a good method to reduce the number of mutants analyzed. It is known that such strains

show changes in the glyoxylate cycle (Finogenova *et al.*, 2008; Karasu-Yalcin, 2012), though such mutations are random and it is impossible to predict the phenotype that will present. Observing the results obtained, the mutants show changes on production profile and on isocitric/citric acid ratio, but some of the mutants did not present a phenotype of interest (lower Isocitric/citric acid ratio or higher citric acid production). In this study, strains were obtained with improved production of citric acid and with a lower ratio of the parent strain, but there were also isolated mutants with lower citric acid concentrations and higher ratios. In Hamissa *et al.* (1982) study mutations in *Candida lipolytica* Y-1095 were induced using UV-irradiation and chemical mutagen (N-methyl-N'-nitro-N-nitrosoguanidine (NG)) and as in this work some of the mutants isolated did not display an improvement on citric acid production. From the seventy seven strains tested only four exhibited an improvement of 75 % - 85 % from the original strain (Hamissa *et al.*, 1982). In other work, *Y. lipolytica* VKM Y-2373 was also exposed to UV-irradiation, NG and a combination of both treatments. From 35 strains tested only 6 presented higher citric acid concentration, the rest of the mutants displayed a similar or low citric acid production (Finogenova *et al.*, 2008). More recently, in a study performed by Karasu-Yalcin (2012), *Y. lipolytica* 57 was exposed to UV-irradiation and a chemical (EMS) to enhance citric acid production. From 18 acetate-negative strains tested only four presented an increase on maximum citric acid concentration than the original strain, the other strains, two presented similar concentrations as the parental strain and the rest produced lower citric acid concentration.

6.3.3 Citric acid production by *Yarrowia lipolytica* W29, UV-75 and UV/EMS-10 in a STR bioreactor

Among the 37 mutant strains previously tested in flasks for citric acid production, *Y. lipolytica* UV-75 and *Y. lipolytica* UV/EMS-10 strains were selected to evaluate the citric acid production profile in bioreactor assays. *Y. lipolytica* UV-75 strain was selected due to the lower isocitric/citric ratio and higher yield compared to *Y. lipolytica* W29. *Y. lipolytica* UV/EMS-10 strain had an isocitric/citric acid ratio similar to *Y. lipolytica* W29, but produced higher citric acid concentration and yield.

Citric acid production by *Y. lipolytica* W29, UV-75 and UV/EMS-10 strains was performed in batch cultures, in a STR bioreactor with crude glycerol as a carbon source. Cellular growth, glycerol

consumption and citric and isocitric acids production profiles of these strains are shown in Figure 6.2. A similar growth profile was observed for all strains tested. The stationary phase was attained around 32 h of culture, but *Y. lipolytica* UV-75 strain reached lower biomass concentration compared to the other two strains (Figure 6.2b). Additionally, crude glycerol consumption profile was similar for W29 and UV/EMS-10 strains (Figure 6.2a, 6.2c) but a lower consumption rate was obtained for UV-75 strain (Figure 6.2b). For all strains, citric and isocitric acid production started when cellular growth reached stationary phase, a consequence of nitrogen depletion (Papanikolaou *et al.*, 2002a; Kamzolova *et al.*, 2005; Morgunov *et al.*, 2013). The production of isocitric acid was similar for all strains, obtaining similar final concentrations (around 1.6 $g \cdot L^{-1}$). According to the results, it is clear that citric acid production was different for each strain. As expected, considering the previous studies in flask, UV/EMS-10 strain achieved the higher citric acid concentration (20.1 $g \cdot L^{-1}$), an improvement of 76 % compared to parental strain *Y. lipolytica* W29. On the other hand, citric acid concentration obtained by strain UV-75 was 11 times lower than the parental strain.

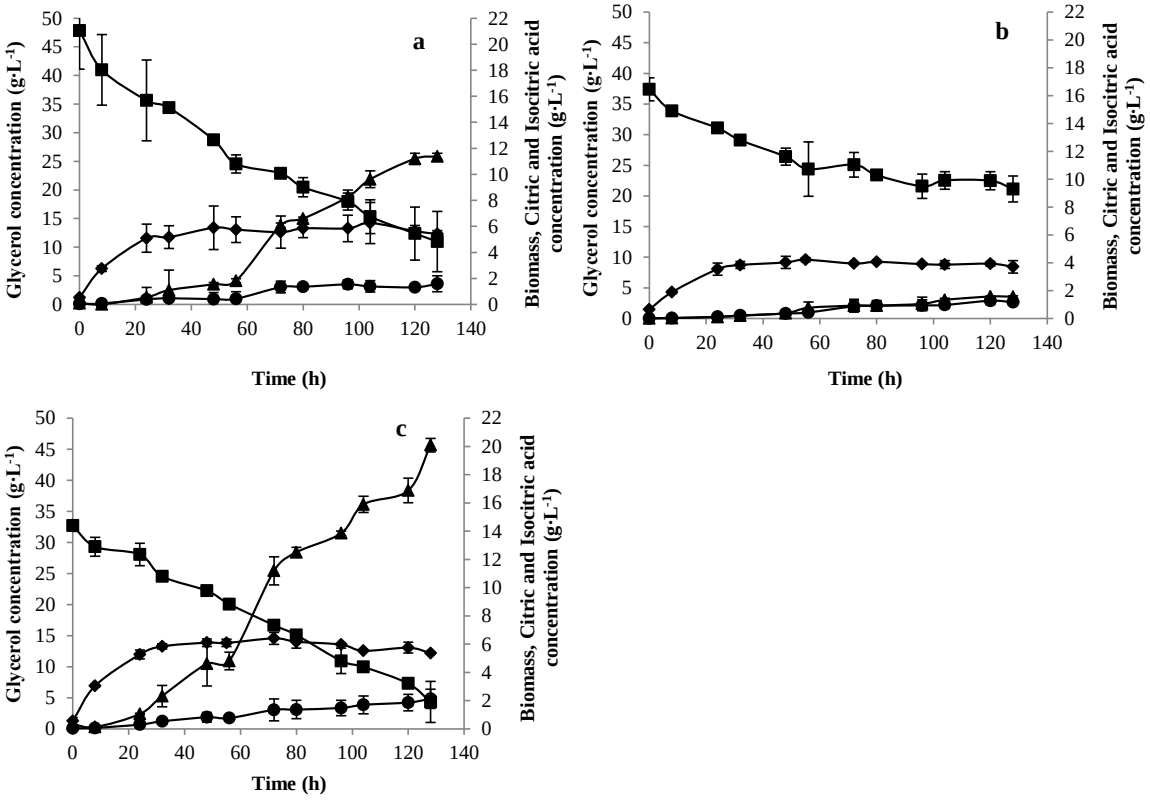

Figure 6.2 Cellular growth (♦), glycerol consumption (■), citric acid (▲) and isocitric acid production (●) profile in batch cultures of *Y. lipolytica* W29 (a) and mutants UV-75 (b) and UV/EMS-10 (c) in a STR bioreactor. The error bar represents the standard deviation of two independent replicates.

A similar maximum specific growth rate was attained for W29 and UV/EMS-10 strains and slightly lower for UV-75 mutant (Table 6.1). However, the biomass yield was higher for UV-75, followed by UV/EMS-10 and parental strain W29 obtained the lowest value. Comparing the citric acid yield and maximum productivity for all strains tested, higher yield and productivity was attained by UV/EMS-10 strain (Table 1). A 2.2- fold improvement on citric acid yield and 60 % on productivity was obtained by UV/EMS-10 strain compared to W29 strain. On the other hand, citric acid yield and maximum productivity obtained by *Y. lipolytica* UV-75 strain were, respectively 3.2 and 6.8 times lower than that attained by the parental strain. Considering the isocitric/citric ratio, no significant differences were observed between W29 and UV/EMS-10 strains, although a slightly lower value was observed to UV/EMS-10 strain. As opposed to what would be expected, the isocitric/citric acid ratio to UV-75 strain was 5.7-fold higher than the parental strain. This alteration on the UV-75 strain ratio may be due to alterations of the mutation probably during the subculture for mass propagation. These alterations may have resulted from the normal cell repair system (Smith-Keary, 1991). Hamissa *et al.* (1982) observed a similar result, after the screening a decrease on citric acid yield was observed. In that work the authors assume a possible further mutation, i.e. a back mutation or a mutation at another site affecting the genes involved in citric acid production (Hamissa *et al.*, 1982).

Table 6.1 Maximum specific growth rate (μ), biomass yield ($Y_{X/S}$), citric acid yield ($Y_{CA/S}$), maximum productivity (P) and isocitric/citric acid ratio (ICA/CA) for parental strain *Y. lipolytica* W29 and mutants UV-75 and UV/EMS-10 growing in crude glycerol batch cultures. The values are presented as average and standard deviation of two independent experiments.

	Y. lipolytica		
	W29	**UV-75**	**UV/EMS-10**
μ (h^{-1})	0.086 ± 0.009	0.066 ± 0.002	0.085 ± 0.004
$Y_{X/S}$ ($g \cdot g^{-1}$)	0.14 ± 0.04	0.19 ± 0.02	0.17 ± 0.01
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.32 ± 0.10	0.10 ± 0.03	0.70 ± 0.06
P ($g \cdot L^{-1} \cdot h^{-1}$)	0.093 ± 0.004	0.0136 ± 0.0002	0.15 ± 0.01
ICA/CA ($g \cdot g^{-1}$)	0.14 ± 0.05	0.8 ± 0.1	0.10 ± 0.03

Y. lipolytica UV/EMS-10 mutant strain, obtained by a combination of UV radiation and EMS treatments seems to be an interesting strain for further studies. The production of citric acid is higher than parental strain, both in flasks and bioreactor assays. The production scale up from the flask to the lab-scale bioreactor led to a 26 % increase of citric acid concentration. The citric acid yield obtained for UV/EMS-10 strain is comparable with other works that used mutant strains in batch cultures and crude glycerol as carbon source. In the literature, yield values using mutant strains from crude glycerol ranged from 0.40 $g \cdot g^{-1}$ to 0.90 $g \cdot g^{-1}$ (Rymowicz *et al.*, 2006; Kamzolova *et al.*, 2011b; Morgunov *et al.*, 2013). In this work, *Y. lipolytica* UV/EMS-10 strain was isolated after an exposure of *Y. lipolytica* W29 to UV-irradiation followed by a treatment with EMS. In Morgunov *et al.* (2013) work, the isolated strain, *Y. lipolytica* NG40/UV7, was also isolated from the combination of treatments and achieved high yield of 0.60 $g \cdot g^{-1}$. Rymowicz *et al.* (2006) studied the production of citric acid by three *Y. lipolytica* mutant stains, 1.31, AWG.7 and K1. The strain with higher yield was *Y. lipolytica* 1.31 with a value of 0.62 $g \cdot g^{-1}$, this strains was isolated after a UV-irradiation treatment. (Kamzolova *et al.*, 2011b), isolated the strain *Y. lipolytica* N15, after a treatment with a chemical mutagen (NG), this train presented very high yield of 0.90 $g \cdot g^{-1}$ in batch cultures using crude glycerol.

6.4 CONCLUSIONS

Y. lipolytica W29 (ATCC 20460) was submitted to UV-irradiation, EMS treatment and a combination of both treatments in order to obtain improved strains with reduced isocitric/citric acid ratio and/or enhanced citric acid production. From the thirty seven acetate-negative strains isolated with different production profiles the strains *Y. lipolytica* UV-75 and *Y. lipolytica* UV/EMS-10 presented the most interesting results. The UV-75 strain displayed an isocitric/citric acid ratio 4 times lower and the UV/EMS-10 strain has a 60 % increase on citric acid concentration and 90 % on yield, comparing with the parental strain. In a lab-scale stirred tank bioreactor assays, *Y. lipolytica* UV/EMS-10 was found to be the most interesting strain with a 76 % enhancement on citric acid concentration and 2.2-fold increase on citric acid yield comparing with the parental strain.

In this work was possible to isolate a better strain (*Y. lipolytica* UV/EMS-10) with higher capacity of producing citric acid than *Y. lipolytica* W29 and this strain presents great interest to be

used in future work. To select new and even better strains with higher citric acid production but mainly with a lower isocitric/citric acid ratio, the *Y. lipolytica* UV/EMS-10 can be submitted to a new mutagenic treatment.

7 GENERAL CONCLUSIONS AND FINAL REMARKS

This chapter presents the overall conclusion and the main outcomes of this thesis.

Taking into account the results obtained in this thesis, the suggestions for future work are also presented.

7.1 GENERAL CONCLUSIONS

Yarrowia lipolytica has been studied as an alternative to *Aspergillus niger* for citric acid production. Under nitrogen-limited conditions, it can produce citric acid from industrial byproducts such as crude glycerol, a byproduct from biodiesel industry available in large amounts. Besides yeast strain, several other factors can affect the citric acid production (maximum concentration, yield and/or productivity) as well as other secondary compounds resulting from the same metabolic pathway. Thus, it is very important to optimize the culture conditions and understand how key factors affect citric acid production by *Y. lipolytica* strains. The studies reported in this dissertation addressed different approaches for the optimization of citric acid production by *Y. lipolytica* from crude glycerol.

The work started with the optimization of culture conditions for citric acid production by two strains of *Y. lipolytica* (W29 (ATCC 20460) and CBS 2073) using an experimental design based in the Taguchi method. pH and oxygen mass transfer rate (OTR) proved to be the factors with more influence on citric acid production in batch cultures for both strains using glycerol as substrate. Moreover, a significant interaction between OTR and salts concentration was found for both strains. Similar citric acid concentrations were obtained for both strains using crude glycerol compared with pure glycerol, validating the possibility of using this byproduct as a low cost carbon source for citric acid production by the *Y. lipolytica* strains used in this study.

OTR was one of the factors with more influence on citric acid production by *Y. lipolytica* from crude glycerol (Chapter 3). Thus, the effect of OTR was evaluated in three different bioreactors, namely a stirred tank (STR), a pressurized and an airlift bioreactor. The raise of oxygen availability led to an increase of citric acid yield and productivity for all bioreactors. In the STR bioreactor the maximum citric acid concentration was attained at a $k_L a$ of 55 h^{-1} or at 60 % of controlled dissolved oxygen concentration (DO). The raise of total air pressure from 1 bar to 2 bar, in the pressurized bioreactor, increased citric acid concentration and yield. For the airlift the highest citric acid concentration was attained at 1.5 vvm. In the range of conditions tested in this work, both bioreactors can be applied for citric acid production process using *Y. lipolytica* cultures and crude glycerol as substrate.

To improve citric acid production and decrease isocitric/citric acid ratio, *Y. lipolytica* W29 was submitted to a mutagenic treatment. From the mutants tested, the higher concentration and

yield of citric acid was obtained by the mutant strain, *Y. lipolytica* UV/EMS-10. This strain has great potential for future work, and can be submitted to new mutagenic treatments to select new and better strains.

7.2 SUGGESTIONS FOR FUTURE WORK

Although the present work brings new insights on the citric acid production, contributing for the optimization of some parameters of relevance for the process, there are still some new ideas for future studies and developments.

In all bioreactors tested, the increase of OTR above the optimal did not influence citric acid production or led to a decrease on citric acid concentration. It would be very interesting to fully understand the role of oxygen in citric acid secretion to the medium. Enzymatic activity and the gene expression of some important enzymes involved in citric acid production, like citrate synthase, citrate lyase, isocitrate lyase and NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase and aconitate hydratase could be performed and a correlation could be obtained to justify the relation with studied parameters and enzymes expression and activity. The high amount of oxygen available can also lead to oxidative stress, which results from the formation of reactive oxygen species (ROS) inside the cell. These ROS can affect all the biological molecules, such as DNA, proteins and lipids. Also, it would be interesting to check possible damages caused by oxidative stress.

The possibility of applying an airlift bioreactor in this process allows the use of immobilized cells. Thus, selection of the better support, optimization of the immobilization methods and culture conditions, and the possibility of reuse the immobilized cells in different batch cultures, could be assessed. The use of continuous mode of operation would be an interesting approach for citric acid production with immobilized cells.

On the other hand, pressurized bioreactors have also great potential of application for CA production, and it would be interesting to implement in this bioreactor type strategies of gradually increasing pressure to obtain high cell density cultures and also to produce CA under fed-batch mode of operation.

In chapter 6, an improved strain was isolated (*Y. lipolytica* UV/EMS-10), which resulted in higher citric acid yield and concentration. Thus, its characterization should be done, and the activity and the gene expression of some important enzymes involved in citric acid production (citrate synthase, citrate lyase, isocitrate lyase and NAD^+- (and $NADP^+$-) dependent isocitrate

dehydrogenase and aconitate hydratase) should be measured. Despite the increased production of citric acid by this mutant strain, isocitric/citric acid ratio remained high. To obtain a strain that produces a minor quantity of isocitric acid, the strain UV/EMS-10 could be submitted to a new treatment with the same mutagens.

Other way to improve the yeast is by genetic modification of the strain. Several enzymes are described as having an important role in citric acid production and accumulation, such as citrate synthase, aconitase hydrate (aconitase), isocitrate lyase, (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2003). In the TCA cycle the enzyme citrate synthase catalyzes the condensation reaction between Acetyl-CoA and oxaloacetate to form citrate and the aconitase hydrate isomerizes citrate into isocitrate. In the glyoxylate cycle isocitrate lyase catalyzes the cleavage of isocitrate into succinate and glyoxylate. Considering the role of these enzymes in the citric acid production, different strategies could be tested:

- Overexpress the *CIT1* gene, that encodes the citrate synthase enzyme, using PO1h strain an auxotrophic strain for uracil which derives from *Y. lipolytica* W29 strain (Nicaud *et al.*, 2002).
- Reduce the strength of the promoter of *ACO1* gene which encodes aconitase hydrate enzyme;
- Overexpress simultaneously the genes *CIT1* and *ICL1*, the gene that encodes isocitrate lyase enzyme from glyoxylate cycle.

Finally, aiming to turn the process as economically attractive as possible in a biorefinery context, strategies may be designed to exploit the various byproducts that are simultaneous produced by the yeast from crude glycerol, such as single cell oil (SCO) and erithrytol.

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